

**Correlation of Protein Homeostasis With Maximum Lifespans  
in Endothermic Vertebrates**

Kurtis Salway

A thesis submitted to the Department of Biological Sciences  
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Faculty of Mathematics and Science  
Biological Sciences, Brock University  
St. Catharines, Ontario

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## **Abstract**

Cellular stress resistance has been shown to be highly correlated with longevity. However, the mechanisms conferring this stress resistance have yet to be identified. Maintenance of protein homeostasis is a critical component of cellular maintenance and stress resistance. Superior protein homeostasis capacities may thus underlie the greater stress resistance observed in longer-lived animals; however, little vertebrate data have been provided supporting this idea. I used two different experimental approaches to test the associations of protein homeostasis capacities with stress resistance and lifespan: 1) a comparison between a large set of vertebrate species with varying body masses and lifespans and 2) a comparison of long-lived Snell dwarf mice and their normal littermates. Protein homeostasis mechanisms including protein degradation activity, protein repair activity and molecular chaperone levels were examined. These measurements were performed in liver, heart and brain tissues, and isolated myoblasts. My results indicated that neither protein degradation nor protein repair were upregulated in association with enhanced stress resistance and longevity in an inter-species and intra-species context. Furthermore, my results did show that there is a positive correlation between molecular chaperone levels and maximum lifespan (MLSP). However, there was no elevation of chaperone levels in the long-lived Snell dwarf mouse, indicating there are other mechanisms linked to their increased lifespan. Therefore, these results suggest that molecular chaperones are involved in increasing animal lifespan in an inter-species context.

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## Chapter 1: Literature Review

### 1.1 Introduction

Animal lifespans are highly dependent on environmental factors including disease, predation and access to adequate nutrition. Although these factors affect the *mean* lifespan of animal populations, one can measure a '*maximum lifespan potential*' for an individual species. This value has often been measured in protected environments (e.g. zoos) in which predation is absent, nutrition should be adequate and disease is aggressively treated (de Magalhães, 2006). Under these conditions, it seems that the maximum lifespan (MLSP) of a species is a true heritable characteristic of a given species. MLSP is considered a generally reliable indicator of species' longevity potential reflecting the 'normal' rate of aging when external factors have been minimized. Recently, AnAge, a free online database, was created and contains detailed MLSP data for thousands of animal species, helping researchers to be more certain that a species' MLSP is accurate (de Magalhães et al., 2005; de Magalhães and Costa, 2009).

Vertebrate endotherms have a wide range of MLSPs from a few years to over a century. However, the cellular and molecular mechanisms responsible for longevity in animals have not yet been precisely determined. Various theories have been proposed to try to explain the differences in lifespan and longevity. The rate of living theory, proposed by Pearl (1928), stated that the faster the metabolic rate of an animal, the shorter their lifespan. However, evidence has since refuted this theory for several reasons (Reviewed in Hulbert et al., 2007). Exercise has been shown to increase metabolic rate and this does not cause a decrease in lifespan in rats (Hollooszy et al., 1985) or humans (Lee et al., 1995). Furthermore, birds and bats have relatively high mass-specific

metabolic rates and they have relatively long lifespans for their body size (Reviewed in Speakman, 2005a; Austad, 2010). Lastly, caloric restriction is known to extend lifespan in several organisms but does not cause a decrease in metabolic rate (Reviewed in Hulbert et al., 2007).

Another prominent theory attempting to explain differences in animal longevity involves cellular senescence. Cells are only able to replicate a specific number of times and then they reach a stage where cell division stops, a phenomenon called senescence. It is believed that the mechanism that causes cellular senescence is the shortening of telomeres. Telomerase is the enzyme responsible for adding telomeric repeats to the end of chromosomes. Therefore, it was thought that an increase in telomerase activity would allow cells to continue to replicate more and have a longer lifespan. However, Seluanov et al. (2007) found that telomerase activity correlates negatively with body mass but there was no correlation with lifespan in several rodent species. This evidence suggests that since large body mass leads to a greater chance of cancer, larger animals must have evolved a mechanism to decrease this cancer risk. This observation does, however argue against increased telomerase activity as a mechanism responsible for inhibiting cellular senescence in vivo and extending lifespan in normal somatic cells (Seluanov et al., 2007).

The disposable soma theory describes somatic maintenance as an energetically costly process, where energy not devoted to cellular repair is used for reproduction (Kirkwood, 1977; Kirkwood et al., 2000). It characterizes aging as an accumulation of cellular damage which can be repaired by cellular mechanisms. It explains that there is an energy tradeoff between somatic cellular maintenance and reproduction. Animals that are more likely to become prey have evolved to expend more energy towards

reproduction (rapid growth and more rapid sexual maturation) and less energy towards cell maintenance, leading to shorter lifespans. However, larger animals, which are less likely to become prey, are able to use more energy on cellular maintenance and less towards reproduction. These animals have longer lifespans, reproduce at a later age, and are able to live past their initial breeding age.

Longevity and lifespan seem to be controlled by a large genetic network involving numerous different genes. Genetic modifications in *Caenorhabditis elegans*, *Drosophila* and *Mus musculus* have shown that several different longevity genes can function to extend lifespan and therefore, each must play some role in the aging process. Interestingly, the protein products of these longevity genes include those involved in pathways that influence metabolism, cell maintenance and repair, or prevent the accumulation of damage to cellular macromolecules (Kuningas et al., 2008).

## 1.2 Oxidative Stress and Damage

Mitochondria are the main cellular source of oxidative stress in the cell in the form of reactive oxygen species (ROS). ROS are formed normally as by-products of aerobic respiration. It has been reported that less than 1% of oxygen consumed by the mitochondria forms superoxide anions ( $O_2^{\cdot-}$ ) (St-Pierre et al., 2002). The formation of  $O_2^{\cdot-}$  comes mainly from the electron transport chain (ETC), where electrons leak from Complexes I and III, and react with  $O_2$ .  $O_2^{\cdot-}$  will react further to produce more damaging forms of ROS including  $H_2O_2$  and peroxynitrite ( $ONOO^-$ ) (Pryor and Squadrito, 1995).

ROS are also generated through other mechanisms in the cell. Xanthine oxidase is able to convert oxygen into ROS through the reaction:  $Xanthine + O_2 + H_2O \rightarrow uric$

acid + H<sub>2</sub>O<sub>2</sub> and has been shown to be implicated in creating oxidative stress (Nishino et al., 2008). Another prominent mechanism of ROS generation is through the enzyme NADPH oxidase. This enzyme is found within neutrophils, and it reduces molecular oxygen into O<sub>2</sub><sup>·-</sup>, which is used in the immune response against foreign microorganisms (Fialkow et al., 2007). ROS can also play an important role in cell signalling pathways, especially in regulation of the cell cycle (Menon and Goswami, 2007).

The Free Radical Theory of Aging postulated by Harman (1956) stated that the accumulation of oxidative damage to proteins, lipids and DNA can result in aging and lead to cell death in organisms. This damage will arise when ROS formation overwhelms the cell's capacity to properly eliminate ROS. Damage to proteins, lipids and DNA are all especially important in leading to aging and therefore, mechanisms to prevent or remove this damage and maintain cellular homeostasis could potentially function to slow the aging process.

### **1.3 Stress resistance and molecular mechanisms conferring longevity**

Kapahi et al. (1999) showed a positive correlation between cellular stress resistance and animal lifespan. A similar trend was observed using an intra-specific model where long-lived individuals were more resistant to some stressors (Murakami et al., 2003; Harper et al., 2007). An interesting observation that was made by Miller (2009) was that lifespan extension does not seem to be conferred through resistance to one stress but to multiple stressors. This led to the idea of multiplex stress resistance, which states that longevity may come from enhanced protection of cellular maintenance

as a whole through several different pathways. Therefore, cellular stress resistance may result in a longer lifespan.

However, research has yet to determine the precise mechanisms that confer longevity. It is believed that possible mechanisms all involve enzymes responsible for cellular maintenance, protection or repair. These ideas stem from the importance and potential vulnerability of lipids, DNA and proteins to oxidative stress. Furthermore, the conserved stress core proteome includes enzymes responsible for ROS neutralization, DNA repair and maintenance of protein homeostasis. Therefore, an increase in activity of the stress core proteome could potentially lead to an increase in stress resistance and lifespan. A recent comparative study by Page et al. (2010) showed that antioxidant enzymes do not co-evolve with longevity in 15 mammalian and avian species, indicating that their activities may not be responsible for increased longevity. Furthermore, a reduction in MnSOD does not reduce lifespan in mice (Van Remmen et al., 2003). Similarly, overexpression of CuZnSOD in mice does not extend lifespan (Huang et al., 2000). Therefore, these results show that the activity of antioxidant enzymes may not be responsible for stress resistance and longevity. However, research on the other aspects of the core stress response is limited.

#### **1.4 20S/26S Proteasome**

Proteins are known to be a major target of ROS, and consequently, it is important to remove any damaged proteins from the cell to prevent aggregation and maintain normal cellular functions (Grune et al., 2004). The proteasome plays a significant role in maintaining cellular protein homeostasis. It is a large protein complex found in the

cytosol and nucleus that is responsible for degradation of damaged or abnormal intracellular proteins. There are several different forms of the proteasome, but the more prominent forms are the 20S and 26S proteasome. The 20S proteasome contains just the catalytic chamber and degrades oxidatively damaged proteins (Reinheckel et al., 1998; Davies, 2001); whereas the ATP-dependent 26S proteasome contains the 20S subunit and a 19S subunit attached at either or both ends. The 19S subunit serves as an activator of the proteasome and is able to recognize ubiquitin-tagged proteins and allow the complex to degrade these proteins (Jung et al., 2009). Ubiquitin is a 76 amino acid peptide that is ligated to damaged proteins by several ubiquitin ligases to target a protein for degradation (Hershko and Ciechanover, 1998).

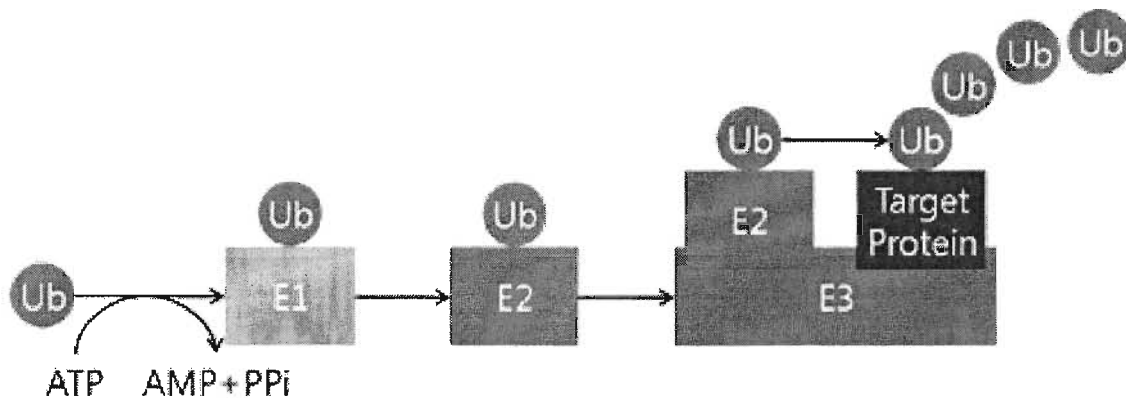


Fig 1.1: The ubiquitin pathway. Ubiquitin ligases add multiple ubiquitin peptides to target protein to tag it for proteasomal degradation (Pickart and Eddins, 2004).

The catalytic chamber is cylinder-shaped and contains 4 stacked heptameric rings each with 7 different subunits. The outer two rings are the alpha rings and the inner two rings are beta rings. The beta rings contain 3 different subunits responsible for the catalytic activity of the proteasome.  $\beta$ -1,  $\beta$ -2 and  $\beta$ -5 subunits are associated with caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing) activity, trypsin-like activity

and chymotrypsin-like activity, respectively. The alpha rings are usually closed off to the cytosol. However, conformational changes in structure caused by exposure to hydrophobic stretches on the protein or ubiquitin-tagging can open the core and allow for proteins to enter for degradation (Davies, 1993; Pacifici et al., 1993).

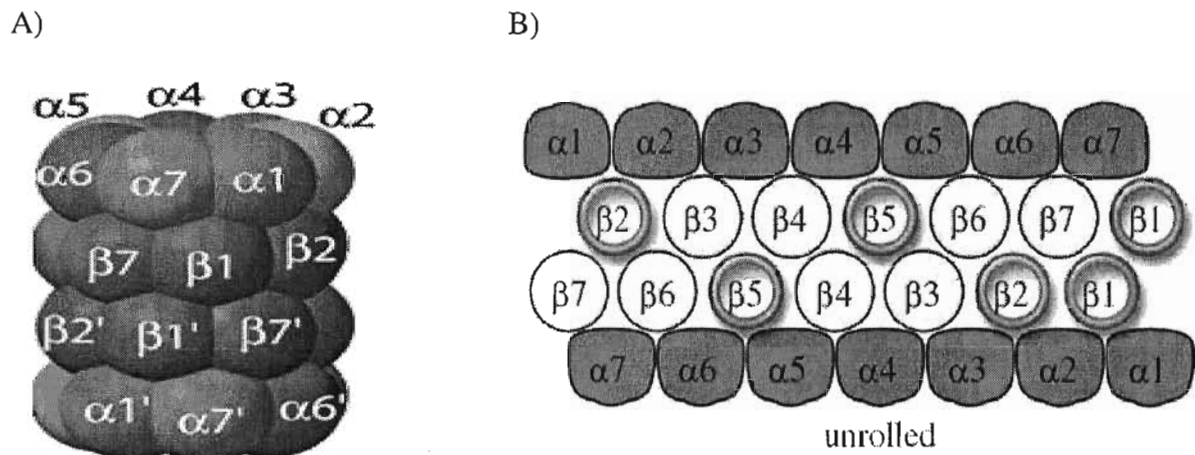


Fig 1.2: The proteasome structure. A) The proteasome catalytic chamber exists as a cylinder containing 4 heptameric rings. B)  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 are the subunits responsible for protein degradation (Rechsteiner and Hill, 2005).

The 20S/26S proteasome is believed to be important during aging due to the increased accumulation of damaged proteins with age, which can lead to aggregation. The proteasome removes these damaged proteins and thus prevents their aggregation, but severe oxidation is able to overwhelm the proteasome (Grune et al., 2001). The formation of cross-linked aggregates can lead to their binding to the proteasome and an inhibition of proteolytic activity, potentially leading to apoptotic or necrotic cell death (Davies, 2001). Furthermore, it has been shown that proteasome activity decreases with age. The formation of these aggregates is thought to be a cause of the development of some neurodegenerative diseases including Parkinson's Disease and Alzheimer's Disease (Martinez et al., 2008).

Genetic modifications of the proteasome have allowed for analysis of their effects on cellular stress resistance. Overexpression of the  $\beta$ -5 proteasome subunit causes an increase in assembled proteasome number and increases its activity in human embryonic fibroblasts (Chondrogianni et al., 2005). In turn, this caused these cells to be more resistant to several stressors, including ethanol and hydrogen peroxide (Chondrogianni et al., 2005). Therefore, these results show that an increase in proteasome activity could increase stress resistance and contribute to longevity.

### **1.5 Protein Repair Enzymes**

Maintenance of protein redox homeostasis is a vital component of cellular stress resistance. The redox state of proteins is crucial to many cellular processes including metabolic, signalling and transcriptional processes. The key player in protein redox homeostasis is the thiol group contributed mainly by cysteine residues. Under normal conditions, the intracellular environment is reduced and the proteins contain many free sulfhydryl groups (Koháryová and Kollárová, 2008). When oxidized, thiol groups can form disulfide bridges and interfere with normal protein functions. Protein repair enzymes function to maintain this redox homeostasis through the regulation of thiol-disulfide exchange on critical cysteine residues. Oxidative stress is believed to be a contributor to the aging process and proteins are a vulnerable target during this stress. Therefore, these protein repair enzymes could maintain protein homeostasis during aging indirectly by removing the effects of ROS on proteins and thus, potentially slowing the aging process (Friguet, 2006). The two major systems predominantly responsible for



maintaining the reduced state of the cellular milieu are the thioredoxin system and the glutaredoxin system.

### 1.5.1 Thioredoxin System

The role of thioredoxin (Trx) is to reduce disulfide bonds to free sulfhydryl groups. In this process, the active site of Trx becomes oxidized and must be reduced for the enzyme to regain its function. Thioredoxin reductase (TrxR) is responsible for reducing Trx's active site using NADPH as an electron donor and allowing this cycle to continue. Therefore, the biological activity of Trx is entirely dependent on TrxR. There are 2 major isozymes of TrxR found in humans and mice; one is found predominantly in the cytosol (TrxR1) and the other in the mitochondria (TrxR2).

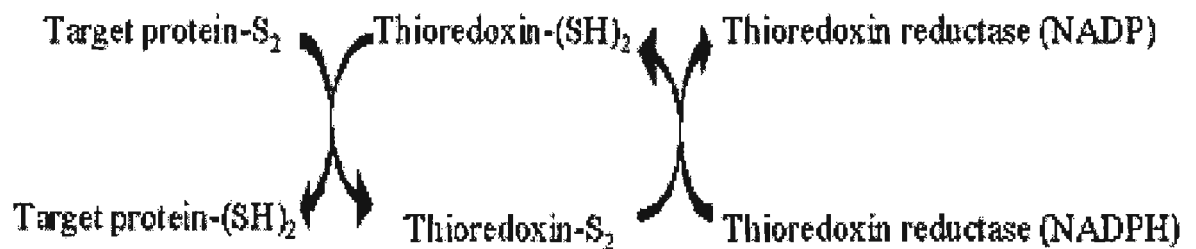


Figure 1.3: Thioredoxin reduces disulfide bonds on target protein and thioredoxin reductase is responsible for reducing the active site of thioredoxin using NADPH as an electron donor (Lu et al., 2007).

Knockout experiments in mice have shown that both forms of TrxR are required for embryonic development, although both show dramatically different phenotypes. Knockout of TrxR1 leads to severe growth retardation and embryonic death at day 9. However, knockout of TrxR2 leads to insufficient heart development, sensitivity to oxidative stress and embryonic death at day 13. (Jakupoglu et al., 2005; Bondareva et al.,

2007). Similarly, *C. elegans* have a reduced MLSP when the TrxR gene is knocked out compared to the wildtype (Miranda-Vizuete et al., 2005).

### 1.5.2 Glutaredoxin System

Glutaredoxin (Grx) is another important protein repair enzyme with three different isoforms present within the cell; one in the cytosol and two in mitochondria (Berndt et al., 2007). Grx is responsible for S-glutathionylation and deglutathionylation of proteins which acts to protect critical thiol groups on amino acid residues, especially cysteine, from oxidation. Similarly to thioredoxin, glutaredoxin's active site must be reduced for it to continue to function. However, it is a non-enzymatic action that acts to reduce the active site with glutathione acting as the oxidizing agent.

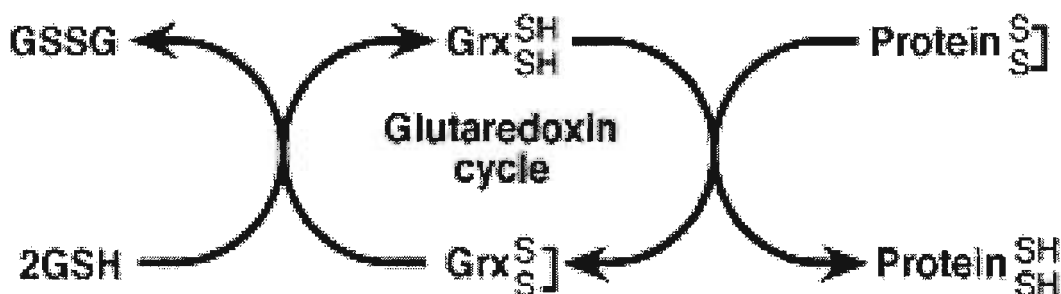


Figure 1.4: Glutaredoxin is responsible for deglutathionylation of target proteins and needs glutathione to reduce its active site for continued function (Trotter and Grant, 2003).

There is evidence that glutathionylation is induced during oxidative stress and can alter the activities of certain proteins ie. actin (Wang et al., 2001) or caspase-3 (Zech et al., 1999). However, Grx is able to reduce these proteins and re-establish their biological activities. Studies have been performed to examine the effects of genetic manipulations

of Grx expression on stress resistance and longevity in both invertebrate and vertebrate models. Lens epithelial cells from Grx gene knockout mice have a reduced resistance to oxidative stress (Lofgren et al., 2008). Furthermore, human lens epithelial cells have an increased resistance to hydrogen peroxide and protection from apoptotic cell death when mitochondrial Grx2 is overexpressed (Lou and Wu, 2009). Therefore, these studies suggest that an increase in Grx expression may cause an elevation of cellular stress resistance and animal lifespan.

### **1.6 Heat Shock Proteins**

The conformational structure of a protein is critical to its catalytic activity and any change in this status can cause protein unfolding and aggregation. Heat shock proteins (Hsps) are a large class of chaperone proteins responsible for assisting in proper protein folding under normal conditions, refolding of proteins unfolded due to stress and the prevention of protein aggregation (Calderwood et al., 2009). Hsps are found in several compartments of the cell including the cytosol, mitochondria and endoplasmic reticulum (ER). Each cellular compartment must be able to maintain its protein homeostasis independently, and therefore, each has its own response to stressful conditions (Ni and Lee, 2007; Truettner et al., 2008). These responses are known as unfolded protein responses (UPR) and occur within each compartment when faced with a protein stressor. When there is an accumulation of unfolded and/or damaged proteins in any compartment, the cell responds by upregulating the transcription of molecular chaperones to alleviate the demands on the already present protein folding machinery.

Since the cytosol, mitochondria and ER have their own responses to protein unfolding, each also has its own compartment-specific chaperones. Hsp70 is one of the more prominent Hsp families with several different members, the majority of which reside in the cytosol. Members of the Hsp70 family are highly conserved and are either constitutively expressed, stress-induced, or both (Daugaard et al., 2007). Hsp60 is the predominant Hsp in the mitochondria and while it is constitutively expressed, it has been shown to be selectively upregulated during the mitochondrial UPR. It has also been shown to play a role in preventing neurodegeneration in human neuroblastoma cells through its participation in the removal of  $\beta$ -amyloid, a contributor to Alzheimer's Disease (Veereshwarayya et al., 2006). In the ER, glucose-regulated protein 78 (GRP78) and 94 (GRP94) are key mediators of the UPR and thus major contributors to the ER stress response. During stressful conditions, transcription of these molecular chaperones increases in an attempt to overcome the accumulation of unfolded proteins (Ni and Lee, 2007).

Hsps and their effects in longevity have been extensively studied using the animal models *Caenorhabditis elegans* and *Drosophila* species. The overexpression of hsp-6 (Yokoyama et al., 2002) or hsp-16 (Walker and Lithgow, 2003) or heat shock factor 1 (HSF-1) (Hsu et al., 2003) has been shown to increase lifespan in *C. elegans*. A similar lifespan extension has been found when Hsps are overexpressed in *D. melanogaster* (Morrow et al., 2010). In studies with murine disease models, overexpression of Hsp70 (Gifondorwa et al., 2007) and HSF-1 (Steele et al., 2008) caused a lifespan extension. However, Vanhooren et al. (2008) showed that overexpression of Hsp70 in normal mice caused shortened lifespan and an increase in tumour formation. Therefore, it seems in

this model that the beneficial stress resistance properties of Hsp70 which could lead to lifespan extension are perhaps overridden by its antiapoptotic properties, potentially leading to an increase in tumorigenesis. Due to the limited number of lifespan studies done in healthy mice with genetically modified Hsp's, there is still a pressing need to further this research in mammals.

### **1.7 Comparative Studies of Longevity**

Two widely used approaches to study the molecular mechanisms of animal longevity are inter-specific and the intra-specific comparisons. The inter-specific approach utilizes comparisons of molecular trait values between naturally shorter- and longer-lived species. Intra-species comparisons typically utilize dietary or genetic manipulations to alter the lifespan of a single species. While both approaches are useful, each has certain inherent limitations.

### **1.8 Inter-species Comparisons**

Inter-species comparisons with a wide range of MLSP are particularly important tools to determine which molecular traits have co-evolved with longevity in animals. Such comparative studies have been used to test relationships between lifespan and mitochondrial ROS production (e.g. Lambert et al., 2007), cellular resistance to numerous chemical and physical stressors (e.g. Kapahi et al., 1999) and antioxidant enzymes (e.g. Tolmasoff et al., 1980; Brown and Stuart, 2007). One issue with many such published studies, however, has been the use of only 2 or 3 species (Ku and Sohal, 1993; Barja and Herrero, 1998). Some of these 2-3 species studies in the literature focus on species which

are extraordinarily long-lived for their body mass, such as bats (Salmon et al., 2009) and naked mole rats (Perez et al., 2009a). However, comparisons of molecular traits within these species to those of mice are difficult to interpret, since in addition to having different lifespans, the two species differ in hundreds or thousands of other respects. For example, differences in proteasome activity found in Salmon et al. (2009) between bats and mice may have arisen from a bat's ability to fly which tends to increase the mass-specific standard metabolic rate. It is therefore not possible to conclude from two species comparisons that any measured differences relate to longevity.

Perhaps the best way to overcome the limitations of two species comparisons is to add more species to the study. To test statistical significance of a correlation between longevity and a specific trait, a minimum of 3 species are required (this arises from the fact that the calculation of degrees of freedom is  $N-2$  and for a statistical correlation, the degrees of freedom cannot be zero; Garland and Adolph, 1994), but studies with a low number of species can also be affected greatly by an increase in outlying data points, leading to biased results and incorrect conclusions. Therefore, it is best to use as many individual species as possible, covering a broad range of the variable in question (i.e. MLSP) in comparative studies.

It is also necessary to consider some of inherent limitations of inter-specific comparisons that must be addressed for accurate interpretations. Firstly, body mass is highly correlated with MLSP. Therefore, any traits that correlate with body mass will also correlate with MLSP. This problem can be addressed by statistically controlling the effects of body mass by correlating the calculated residuals from the regression line (vertical distance from regression line) of body mass (Fig 1.1A) and the trait of interest

and the residuals of the regression line of body mass and MLSP (Fig 1.1B) (reviewed in Speakman, 2005).

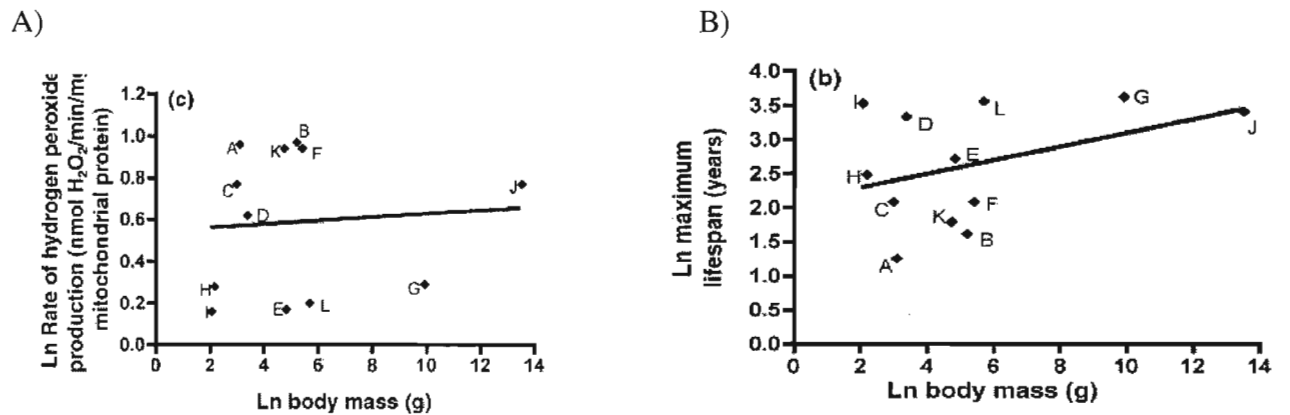


Fig 1.5: The analysis of residuals using the regression lines. A) The relationship between the trait of interest and body mass is shown whereas B) shows the relationship between MLSP and body mass (Lambert et al., 2007)

The second limitation is due to all extant animals being products of a shared evolutionary history, which makes data points in the correlation (i.e. species) non-independent.

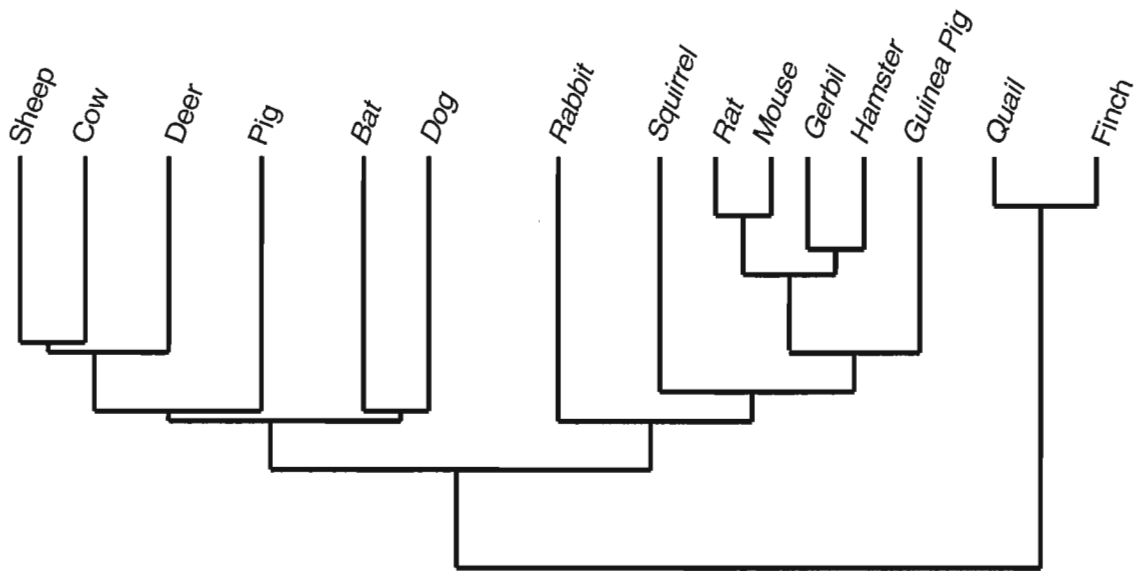


Fig 1.6: Phylogenetic tree showing branch lengths that can be used for FIC analysis.

Independence of data points is a prerequisite for correlational analyses. A solution to this problem is to transform the raw data into Felsenstein's independent contrasts (FIC) as outlined in Speakman (2005). Although the species data are not independent, the contrasts or difference between pairs of species in the phylogeny are independent and this allows for reliable statistical analysis. Therefore, it can ensure that any differences in a specific molecular trait between species are due to the product of evolution and not due to a shared phylogenetic history (Felsenstein, 1985). This requires having a phylogeny based on molecular sequences of specific traits or genomes.

### **1.9 Intra-species Comparison**

Intra-species comparisons use a single representative species to test potential longevity genes. Many of the intra-species studies performed involve the over-expression or under-expression of a gene through genetic manipulation. Even though this approach benefits from the reduction in genetic variability and ability to strictly control dietary and environmental conditions, some inherent weaknesses must be noted. Many intra-specific studies use highly inbred animal models such as *C.elegans*, *D. melanogaster* or *Mus musculus*. Miller et al. (2002) showed that highly inbred laboratory mice have evolved to promote early reproductive maturity and large litter sizes compared to wild-type mice. Interestingly, it is possible that selection for these desirable characteristics has removed normal wild-type alleles that function to slow aging. Thus, a specific genetic manipulation in a laboratory mouse that causes an increase in lifespan may simply be restoring the original genetic background compromised through inbreeding. Inbreeding has also led to inter-laboratory variability in strains of mice (Harrison et al., 2009).



Furthermore, it is largely unknown to what extent these genetic mutations will affect animal fitness in natural populations and whether these candidate genes contain variation in natural populations, which could contribute to lifespan variation. This is important because a candidate gene may not contain variation in nature and would have no effects on lifespan (Kuningas et al., 2008).

Another discrepancy that may exist in using genetic modifications is that a certain gene modification may have an effect in one species but not in another. This can be especially true when comparing invertebrate and vertebrate animal models. For instance, it is possible to look at lifespan extension in mutants that affect the insulin/IGF-1 pathways. *C. elegans* live exceptionally longer when they have a mutation in the *daf-2* gene, which encodes for insulin-like receptor, and leads to an increased level of Hsps (Hsu et al., 2003). However, Snell dwarf mice have a mutation that also affects their insulin/IGF-1 signalling and extends lifespan, but they have no increase in Hsp expression (Swindell et al., 2009).

Thus, there are some limitations to using genetic modifications within a single model organism as a tool to identify longevity genes, but it can be still be a valuable tool. The combined use of an intra-specific and inter-specific approach may be the best way to accurately verify if a specific gene of interest is truly a 'longevity gene'.

### **1.10 The Snell Dwarf Mouse as a Model of Longevity**

The Snell dwarf mouse has a point mutation within the *Pit1* gene which encodes a transcription factor that is necessary for the proper development of the anterior pituitary gland. This mutation leads to secondary deficiencies in growth hormone (GH), thyroid-

stimulating hormone (TSH), prolactin (PRL), and insulin-like growth factor 1 (IGF-1) levels. As adults, they attain only one-third the weight of their normal littermates. In addition, they have a reduced metabolic rate (ie. reduced core body temperature and mass-specific metabolic rate). Interestingly, these mice have also been shown to have lifespans ~50% longer than their normal littermates under optimal housing conditions (Reviewed by Flurkey et al., 2002).

Enhanced cellular stress resistance has been well characterized in Snell dwarf mice. Dermal fibroblasts from Snell dwarf mice are more resistant to oxygen-induced cellular senescence (Maynard and Miller, 2006), exposure to UV, hydrogen peroxide and paraquat (Murakami et al., 2003). Stress resistance in these animals appears to involve numerous protective mechanisms, including ROS neutralization, protein and DNA repair. However, these mechanisms have not been studied in great detail in Snell dwarf mice.

### **1.11 Myoblasts**

Muscle-specific stem cells or satellite cells are responsible for growth, repair and maintenance of skeletal muscle. They are quiescent mononucleated cells that are found between the basal lamina and the sarcolemma of the muscle fibers. In response to a muscle injury, muscle-specific stem cells are activated and began to proliferate into myogenic precursor cells or myoblasts. Myoblasts are muscle precursor cells that are committed to becoming skeletal muscle (Tedesco et al., 2010).

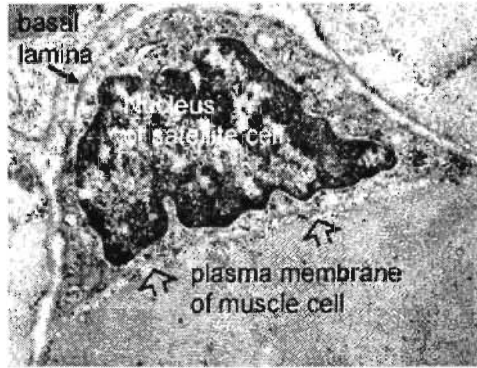


Figure 1.7. Muscle-specific stem cells are quiescent cells located between the basal lamina and the plasma membrane of the muscle fibers. (Fontana et al., 2009)

Aging is associated with a significant reduction in muscle regenerative capacity and increased vulnerability to age-related pathologies. Therefore, the function of muscle-specific stem cells should appear to be important in aging and animal longevity (Bigot et al., 2008). There are two possible roles that satellite cells or myoblasts may play in aging. Firstly, a larger stem cell pool will allow for more muscle regeneration and muscles will be able to function normally for much longer. Therefore, it is possible that longer-lived animals have more stem cells and thus, more protection from damage caused by aging. Secondly, the more stress resistant the stem or progenitor cells themselves are, the longer they will be able to remain functional and the greater the regenerative capacity of these cells as an animal ages.

The use of myoblasts in a comparative study has several advantages over using organ tissue homogenates. A criticism of using tissues from different species from several different sources is that it is virtually impossible to control the environment and diets, especially of the wild animals. These conditions could lead to stressful conditions and measurements of basal levels of a protein could be inaccurate. However, culturing myoblasts allows for precise control over the immediate cellular environment including

nutrient levels. Another advantage of using myoblasts is that one can actually stress the cells and measure the induction of stress proteins. Subjecting large animals to a stressor (e.g. paraquat) would be both extremely difficult and potentially unethical. The third advantage is the regenerative capacity of these myoblasts and their potential role in slowing the aging process.

### **1.12 Hypotheses and Approach**

Protein homeostasis is critical for cellular maintenance and stress resistance. However, proteins are a vulnerable target for stress including oxidative and thermal stress. Exposure to oxidative stress can lead to protein damage, altering protein secondary and tertiary structure and potentially leading to aggregation. To determine if protein degradation and protein repair play a role in enhanced cellular stress resistance and increased longevity, the activities of the 20S/26S proteasome and protein repair enzymes, thioredoxin reductase and glutaredoxin were measured in 1) liver, heart and brain tissues of 13 mammalian species and 2 avian species; 2) heart and brain tissues from Snell dwarf and normal mice and 3) myoblasts isolated from 9 different species.

Prevention of protein unfolding due to cellular stress may also play a valuable role in maintaining protein function and protein homeostasis. To determine if levels of molecular chaperone proteins correlated positively with animal longevity, protein levels of four different chaperones from different compartments of the cell were measured in 1) liver, heart and brain tissues of 13 mammalian species and 2 avian species; 2) heart and brain tissues from Snell dwarf and normal mice and 3) myoblasts isolated from 9 different species.

I hypothesize that, if protein homeostasis contributes to longevity, the cellular capacities for protein degradation and repair, as well as levels of molecular chaperones, should correlate positively with lifespan in both the inter-species and intra-species comparative models.

## **Chapter 2: Enhanced protein repair and recycling are not correlated with longevity in 15 vertebrate endotherm species**

### **Hypothesis**

Animal longevity correlates positively with cellular stress resistance.

Furthermore, maintenance of protein homeostasis is an integral part of stress resistance, and therefore, protein degradation and protein repair activities should correlate positively with longevity.

### **Objective**

The primary objective of this project was to determine if protein degradation and protein repair enzymes play a role in longevity in an inter-specific and intra-specific context.

### **Publications of results**

**Salway KD, Page MM, Faure PA, Burness G, Stuart JA.** Enhanced protein repair and recycling are not correlated with longevity in 15 vertebrate endotherm species. *Age (Dordr)*. 33: 33-47, 2010.

### **Contributions**

I performed all experiments, phylogenetic and statistical analysis and chapter write-up.

## 2.1 Introduction

Vertebrate endotherms display a wide range of maximum lifespans (MLSP), from several years to over a century. Yet aging processes appear strikingly similar among endotherm species, suggesting it may stem from common cellular and molecular mechanisms. It follows that the evolution of increasing MLSP may occur via selection of common key molecular characters; however, we have a limited understanding of what these molecular characters are.

Enhanced resistance to exogenous stressors appears to have co-evolved with longevity (reviewed in Robb et al. 2009). Interspecies comparisons indicate that, with some exceptions, longer-lived species have increased cellular stress resistance (Kapahi et al. 1999; Ogburn et al. 2001; Miller et al. 2010), and comparisons among mice harbouring single gene mutations that increase lifespan also reveal this trend (Holzenberger et al. 2003; Salmon et al. 2005; Maynard and Miller 2006; Bokov et al. 2009). A key observation is that inter- and intra-species differences in cellular stress resistance are present in young adults, suggesting that longevity is associated with enhanced stress resistance over the lifespan rather than becoming evident only with advanced age. The positive correlation between cellular stress resistance and MLSP is consistent with the disposable soma theory of lifespan, which suggests the extent of the investment made in cellular maintenance will be determined by selective pressures to extend lifespan (Kirkwood et al. 2000).

Maintenance of protein homeostasis is a critical component of cellular maintenance and defence against stress (Morimoto 2008). Proteins are susceptible to the oxidative stress that arises directly from exposure to oxidants, or secondarily due to the

disruption of metabolic redox pathways. Exposure to oxidative stress can lead to oxidative protein damage that directly alters protein structure, leading to dramatic decreases in the activity and/or aggregation of partially denatured proteins. Aberrant protein aggregation in brain tissue is a key feature of the major neurodegenerative disorders associated with aging (Martinez et al. 2008). To maintain protein function in the face of oxidative stress, cells utilize 'protein repair' enzymes, capable of maintaining protein redox homeostasis through the regulation of thiol-disulfide exchange on critical residues, particularly cysteine. Important protein repair enzymes include thioredoxin, thioredoxin reductase, and glutaredoxin. Thioredoxin (Trx) is responsible for reducing disulfide bonds on proteins, and thioredoxin reductase (TrxR) reduces the oxidized active site of Trx to maintain its maximal activity. TrxR therefore maintains the thioredoxin cycle - an important component of cellular resistance to oxidative stress (Arner 2009). *Caenorhabditis elegans* with a TrxR null gene have a reduced mean and maximal lifespan compared to wild type (Miranda-Vizuet et al. 2005). Glutaredoxin (Grx) is another important enzyme for maintaining protein function during oxidative stress by catalyzing S-glutathionylation and deglutathionylation of proteins, protecting thiol groups from oxidation. Lens epithelial cells of *Grx* gene knockout mice show decreased resistance to oxidative stress (Lofgren et al. 2008).

Although protein repair enzymes play a significant role in protein homeostasis, the majority of protein damage cannot be reversed or repaired, and so compromised proteins must be removed to prevent their aggregation and interference with normal cellular activities (Grune et al. 2004). The 20S/26S proteasome is a multisubunit proteolytic complex found in the cytosol and nucleus that performs this task. The 20S



proteasome degrades oxidatively damaged proteins (Reinheckel et al. 1998; Davies 2001), whereas the 26S proteasome, which is composed of the core 20S barrel with regulatory cap regions on either end, recognizes and degrades ubiquitin-tagged proteins that may or may not be damaged (Jung et al. 2009). In addition to a global role in damaged protein degradation, the nuclear 26S proteasome also degrades ubiquitylated RNA polymerase II associated with transcriptional arrest at sites of DNA damage (Daulny and Tansey 2009), and ubiquitin-tagged apoptotic proteins thus preventing cell death (Bader and Steller 2009). Ubiquitin is a 76 amino acid peptide that is ligated to damaged proteins through the catalytic activity of ubiquitin ligases, thus targeting them for degradation (Hershko and Ciechanover 1998).

In this study we test the hypothesis that longer-lived species have superior protein homeostasis mechanisms, manifesting as enhanced protein repair and proteasomal degradation activities. To address this, activities of thioredoxin reductase, glutaredoxin, and the 20S/26S proteasome were measured in liver, heart and brain of 15 vertebrate endotherm species with MLSPs ranging from 3 to 30 years (Table 1). Steady state ubiquitin levels were measured by western blot in eight species for which ubiquitin's amino acid sequence was known to be 100% conserved. By using this broad comparative approach with 15 species we avoid the erroneous assumption that all between-species differences in these measured activities are attributable to differences in MLSP rather than body mass, metabolic rate, or another unknown parameter (reviewed by Speakman 2005). We use analysis of residuals to differentiate associations with body mass from those with MLSP or metabolic rate. Felsenstein's independent contrasts (Felsenstein 1985) are also employed to address problems associated with non-independence of data

points (i.e. species) due to shared evolutionary history. Finally, we also measure these same activities in heart and brain of young adult long-lived Snell dwarf mice and their age-matched normal littermates to investigate whether increased lifespan is associated with enhanced protein homeostatic mechanisms in this intra-specific model of longevity.

## **2.2 Materials and Methods**

### ***2.2.1 Materials***

Chemicals were purchased from Bioshop (Burlington, ON, Canada) and Sigma Aldrich (Oakville, ON, Canada; including Fluka and Caledon). BioRad protein dye was purchased from BioRad Laboratories (Hercules, California, US). Proteasome peptide Suc-LLVY-AMC, proteasome inhibitor MG-132 and AMC calibration standard were purchased from Enzo Life Sciences (Plymouth Meeting, Pennsylvania, US). Anti-ubiquitin primary antibody (P-4D1) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, US). Anti-mouse (goat) secondary antibody was purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, US). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA).

### ***2.2.2 Animals***

Between 3 and 6 individuals of each of 13 mammalian and 2 avian species were used in this study (Table 1). Species were selected on the bases of: (1) phylogenetic position; (2) MLSP; (3) routine diet and (4) availability. With respect to phylogenetic position, we confined our analysis to species with a similar overall body plan and physiology, i.e. vertebrate endotherms. Within this constraint, we sampled species within

and between orders. We also attempted to maximize the range of MLSP represented by species included in the study. We also included species with exceptionally long lifespans for their body mass (big brown bat, zebra finch). The nature of the study meant that we were unable to directly control for differences in diet specific to a given species; however, our species collection includes graminivores, nectivores, insectivores, omnivores and carnivores and no trends were noted that related to dietary preference. We also included wild-caught species where possible to control for domestication effects. All animals were healthy young adults, and in most cases the exact age and sex of the animal were known.

### ***2.2.3 Tissue collection***

Normal C57BL mice were euthanized by cervical dislocation following which brain, heart and liver tissues were immediately excised, flash frozen in liquid nitrogen and stored at -80°C. Snell normal and dwarf mice were anaesthetized with isoflurane, injected with a mixture of 50 µg/g ketamine and 5 µg/g xylazine before tissue excision within 30 - 45 min post-mortem. These tissues were flash frozen in liquid nitrogen and stored at -80°C. Norway rats were euthanized with sodium pentobarbitol sodium injection (Euthanyl<sup>®</sup>; 2 µg/g body wt), and brain, heart, and liver tissues were removed and flash frozen in liquid nitrogen and stored at -80°C. Tissues from 13-lined ground squirrels were collected at the University of Western Ontario (London, ON, Canada) as described in Page et al. (2009). Active (i.e. non-hibernating) animals were euthanized by Euthanyl overdose (270 mg/ml, 0.2 ml/100 g body wt), brain, heart and liver tissue were rapidly removed, flash-frozen in liquid nitrogen, and stored at -80°C. Big brown bats, collected from the wild and housed in a captive research colony at McMaster University

(Hamilton, ON, Canada), were euthanized using 0.6 mg/g body wt sodium pentobarbitol followed by decapitation. Bat brain, heart and liver tissues were excised and flash frozen in liquid nitrogen and stored at -80°C. Zebra finch and Japanese quail tissues were collected at Trent University (Peterborough, ON, Canada). Birds were euthanized with 2.0 (finch) or 0.6 (quail) mg/g body wt sodium pentobarbitol (Euthasol<sup>®</sup>), followed by decapitation. Bird brain, heart and liver tissues were removed and flash frozen in liquid nitrogen and stored at -80°C. Guinea pigs were euthanized, and heart, brain and liver tissues rinsed in 1X PBS and immediately frozen in liquid nitrogen and shipped to Brock University (St. Catharines, ON, Canada) on dry ice. Similarly excised tissues from euthanized dogs, hamsters and gerbils were immediately frozen in liquid nitrogen and shipped to Brock University (St. Catharines, ON, Canada) on dry ice. Tissue from domesticated livestock was collected at a local abattoir during normal processing, with the exception of rabbits which were collected during processing from a local farmer. In all cases, brain, heart and liver tissues were collected from these animals within approximately 30 min post mortem. Due to the size of organs from domesticated livestock and rabbits, tissue samples were isolated from the following locations: brain cortex, right cardiac ventricle, and tip of the left liver lobe. Tissues were frozen on-site in dry ice then brought to Brock University (St. Catharines, ON, Canada) where they were stored at -80°C. Fresh white-tailed deer tissue (collected within 1 h post mortem) was obtained by hunters in Cobden (ON, Canada), following which brain, heart and liver tissue was collected and frozen at -20°C for one month, and then transferred to Brock University (St. Catharines, ON, Canada) where it was stored at -80°C. Tissue sampling sites were unspecified for the white-tailed deer samples.

**Table 2.1: Sex, age, mass and maximum lifespan (MLSP) of the mammalian and avian species. Species MLSP data are from AnAge (de Magalhaes et al., 2005).**

| Common Name                | Scientific Name                      | Number and Sex | Approximate Age | Body Mass (kg) | MLSP (yrs) | Source  |
|----------------------------|--------------------------------------|----------------|-----------------|----------------|------------|---|
| <b>Mammalia</b>            |                                      |                |                 |                |            |   |
| Snell mouse (DW/J)         | <i>Mus musculus</i>                  | 6 F (+/dw)     | 6-7 months      | 0.02996        | 2.7        | Jackson Laboratories (Bar Harbor, ME, USA)                        |
|                            |                                      | 6 F (dw/dw)    | 6-7 months      | 0.0096         | 3.9        |   |
| C57BL/6Ncr1BR mouse        | <i>Mus Musculus</i>                  | 4 F<br>4 M     | 4 months        | 0.0276         | 3.5        | Charles River (Wilmington, MA, USA)                               |
| Norway rat                 | <i>Rattus norvegicus</i>             | 6 M            | 3 months        | 0.55           | 5          | Charles River (Wilmington, MA, USA)                               |
| 13-lined ground squirrel   | <i>Spermophilus tridecemlineatus</i> | 8 F<br>3M      | Unknown         | 0.2050         | 7          | University of Manitoba Field Research Station (Carmen MB, Canada) |
| Rabbit                     | <i>Oryctolagus cuniculus</i>         | 4 F<br>1 M     | 3.5 months      | 2.6            | 11.8       | Local abattoir (Fort Erie, ON, Canada)                            |
| Guinea Pig                 | <i>Cavia porcellus</i>               | Unknown        | Unknown         | 1.05           | 12         | Rockland (Gilbertsville, PA, USA)                                 |
| Big brown bat              | <i>Eptesicus fuscus</i>              | 3 F<br>2 M     | 6-12 months     | 0.025          | 19         | McMaster University Bat Laboratory (Hamilton, ON, Canada)         |
| Sussex Sheep               | <i>Ovis aries</i>                    | 5 F            | 6-12 months     | 29.5           | 19.6       | Local abattoir (Fort Erie, ON, Canada)                            |
| White-tailed deer          | <i>Odocoileus virginianus</i>        | 3 M            | 2.5-3.5 years   | 110            | 21.6       | Local hunters (Cobden, ON, Canada)                                |
| Domestic Dog               | <i>Canis familiaris</i>              | 3 M            | 2-8 years       | 11             | 24         | Equitech-Bio, Inc. (Kerrville, TX, USA)                           |
| Yorkshire/ Hampshire Pig   | <i>Sus scrofa</i>                    | 2 F<br>3 M     | 6 months        | 99.9           | 27         | Local abattoir (Fort Erie, ON, Canada)                            |
| Black angus/ - Charlet Cow | <i>Bos taurus</i>                    | 4 F<br>1 M     | 1.5-2.5 years   | 379.5          | 30         | Local abattoir (Fort Erie, ON, Canada)                            |
| Human                      | <i>Homo sapiens</i>                  | 2 F<br>2 M     | 26-37           | 68             | 122        | Zenbio (Research Triangle Park, NC, USA)                          |
| Mongolian Gerbil           | <i>Meriones unguiculatus</i>         | Unknown        | 4-8 months      | 0.070          | 6.3        | Equitech-Bio, Inc. (Kerrville, TX, USA)                           |
| Syrian Hamster             | <i>Mesocricetus auratus</i>          | Unknown        | 4.8 months      | 0.130          | 3.9        | Equitech-Bio, Inc. (Kerrville, TX, USA)                           |
| <b>Aves</b>                |                                      |                |                 |                |            |   |
| Japanese quail             | <i>Coturnix japonica</i>             | 2 F<br>2 M     | 4-5 months      | 0.264          | 6          | Cro Quail Farms Inc (St. Anne's, ON, Canada)                      |
| Zebra finch                | <i>Taeniopygia guttata</i>           | 3 F<br>3 M     | 1 year          | 0.0166         | 14.5       | Trent University Animal Facility (Peterborough, ON, Canada)       |

#### ***2.2.4 Preparation of Tissue Homogenates***

Brain, heart and liver tissues were homogenized using a PowerGen 125 Homogenizer (Fisher Scientific, Ottawa, Ontario, Canada) in ice cold lysis buffer (50mM HEPES, 20mM KCl, 0.1mM EDTA, 1mM DTT, 5% glycerol and 0.1% NP40) at full speed for 3 cycles of 10 seconds with 10 seconds rest between each cycle. Following homogenization, samples were centrifuged at 10000 x g for 20 min. Protein concentration was determined using the Bradford technique with a BioRad protein assay kit and homogenates were aliquotted and stored at -80°C.

#### ***2.2.5 20S and 26S Proteasome Activity Assays***

Assays for 20S and 26S proteasome activities were performed as described by Rodgers and Dean (2003) at room temperature (23°C) using a Varian spectrofluorometer. Conditions for the 20S proteasome assay were 25mM HEPES pH 7.5, 0.5mM EDTA, 25µM peptide and 100µg sample. The peptide Suc-LEU-LEU-VAL-TYR was used to measure the chymotryptic activity of the proteasome. Activity of the 26S proteasome was measured in the same manner except samples were pre-incubated in 5mM ATP for 10 min (ATP was retained throughout the activity assay) to stimulate 26S proteasome activity. Both assays were conducted with an excitation wavelength of 360nm and an emission detection wavelength of 460nm. Activities were determined by measuring the release of the 7-amino-4-methylcoumarin (AMC) fluorophore from the peptide-AMC complex for 15 min. Parallel assays were run with 0.2mM of the specific proteasome inhibitor MG-132 to determine non-proteasomal AMC release. This non-specific activity was minor, but was subtracted from the rate measured in the absence of inhibitor. The standard curve for proteasome calculations used a serial dilution of 8µM AMC

calibration standard to obtain the relationship between arbitrary fluorescence units (AFU) and [AMC]. Specific proteasome activity was calculated by interpolation from the standard curve according to the equation:

Activity (pmol/hr/ $\mu$ g) = slope x (AFU/min) x conversion factor ( $\mu$ M/AFU) x vol x (60min/hr) per mg protein, where the conversion factor was 1/slope of the AMC standard curve.

### ***2.2.6 Quantification of Protein Ubiquitylation***

Brain, heart and liver tissue homogenates (50 $\mu$ g) were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membranes were incubated in a blocking solution, consisting of 5% skim milk in 1x PBS, for 45 min at room temperature. Following blocking, the membranes were incubated with anti-ubiquitin antibody (1:250) overnight at 4°C in solution containing 5% skim milk in 1x PBS-T and then incubated with anti-mouse secondary antibody for 1 hr at room temperature. The membranes were scanned using an Odyssey infrared visual system (LI-COR Biosciences). Ubiquitin level quantification was performed using Odyssey Imaging Software, Version 1.0. The intensity for each sample was compared to an internal standard control which simultaneously resolved within every gel. Band intensity represents intensity of entire lane from top to bottom of membrane.

### ***2.2.7 Thioredoxin Reductase Activity Assay***

The activity of TrxR was assayed essentially as in Arner et al. (1999) in a solution containing 50mM potassium phosphate buffer, 1mM EDTA, 0.2mg/mL, 0.2mM NADPH and protein sample (50-100 $\mu$ g). The reaction was initiated by the addition of 2.5mM DTNB and measured at 412nm. Parallel assays were performed with 0.5mM of the

specific thioredoxin reductase inhibitor dichloronitrobenzene (DCNB) to determine non-proteasomal AMC release. Although, the non-specific activity was negligible, this activity was subtracted from the rate measured in the absence of inhibitor.

#### ***2.2.8 Glutaredoxin Activity Assay***

Grx activity was assayed essentially as described in Kumar and Holmgren (1999). The reaction buffer contained 0.1M Tris-HCl, 2mM EDTA, 100µg/mL BSA, 0.4mM NADPH, 1U/mL glutathione reductase, 10mM GSH and 50µg of protein. A background change in absorbance was monitored prior to the addition of 2-hydroxydisulfide, which initiated the reaction.

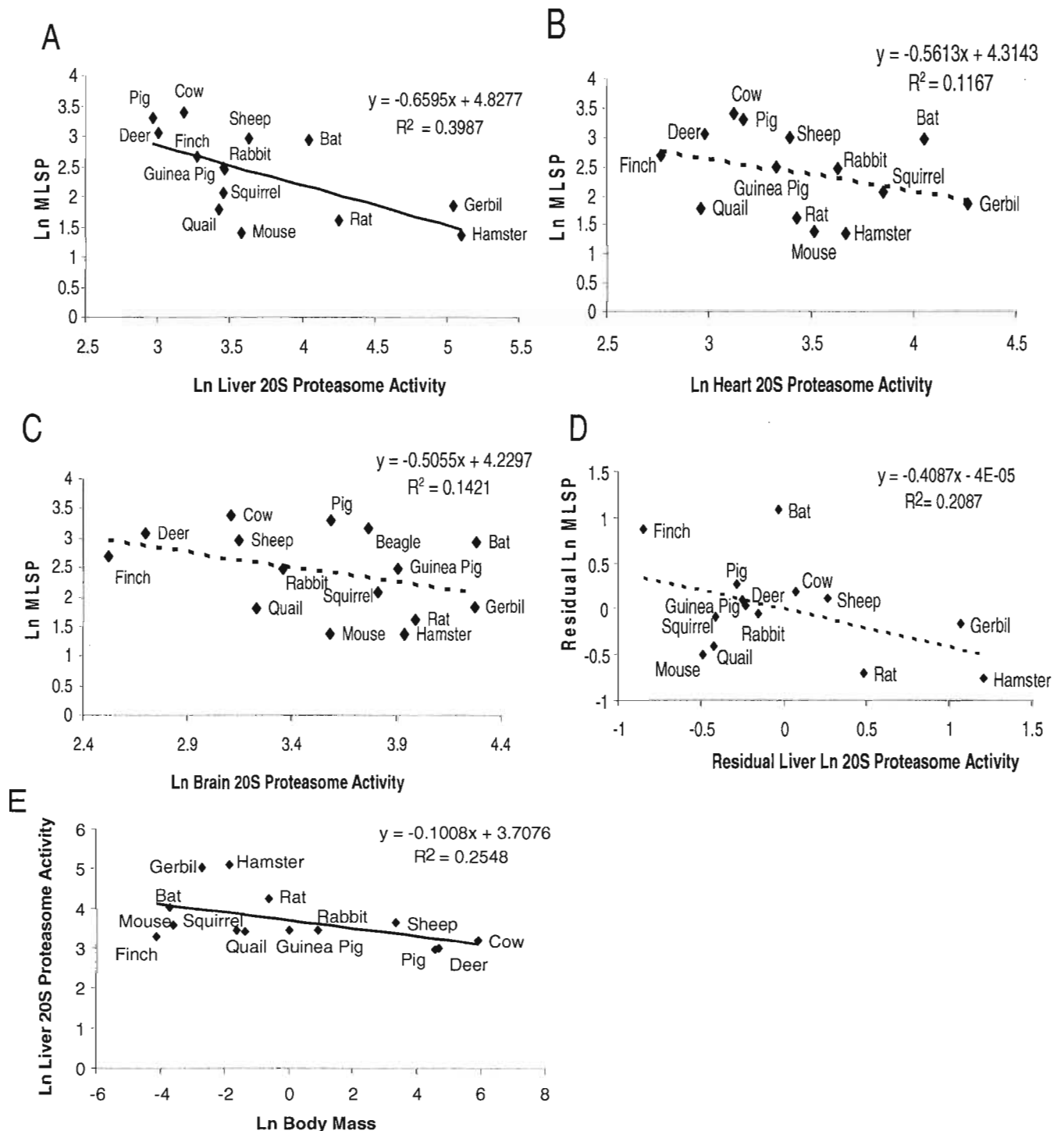
#### ***2.2.9 Statistical Analyses***

Raw data were natural logarithm (Ln)-transformed prior to correlation analyses. Residuals were calculated from simple linear regression of the dependent variable of interest on body mass to remove the confounding effect of body mass. Significance of the coefficient of correlation was used to determine if the line was different from horizontal, and significance was based on t-values for one-tailed tests. A p-value of 0.05 was considered significant. Felsenstein's phylogenetically independent contrasts (Felsenstein 1985) were calculated using PDAP. The phylogenetic tree was constructed from four phylogenies from which branch length estimates were taken (Stuart et al. 2002; Springer and Murphy 2007; Gorbunova et al. 2008; Hackett et al. 2008; Prasad et al. 2009); however, the analysis was repeated with all branch lengths set to one, as with a speciation model of character change (Price 1997). Statistical analyses within the intra-species context (Snell dwarf mice) used a two-tailed t-test to compare normal mice and Snell dwarf mice. A p-value of 0.05 was considered significant.



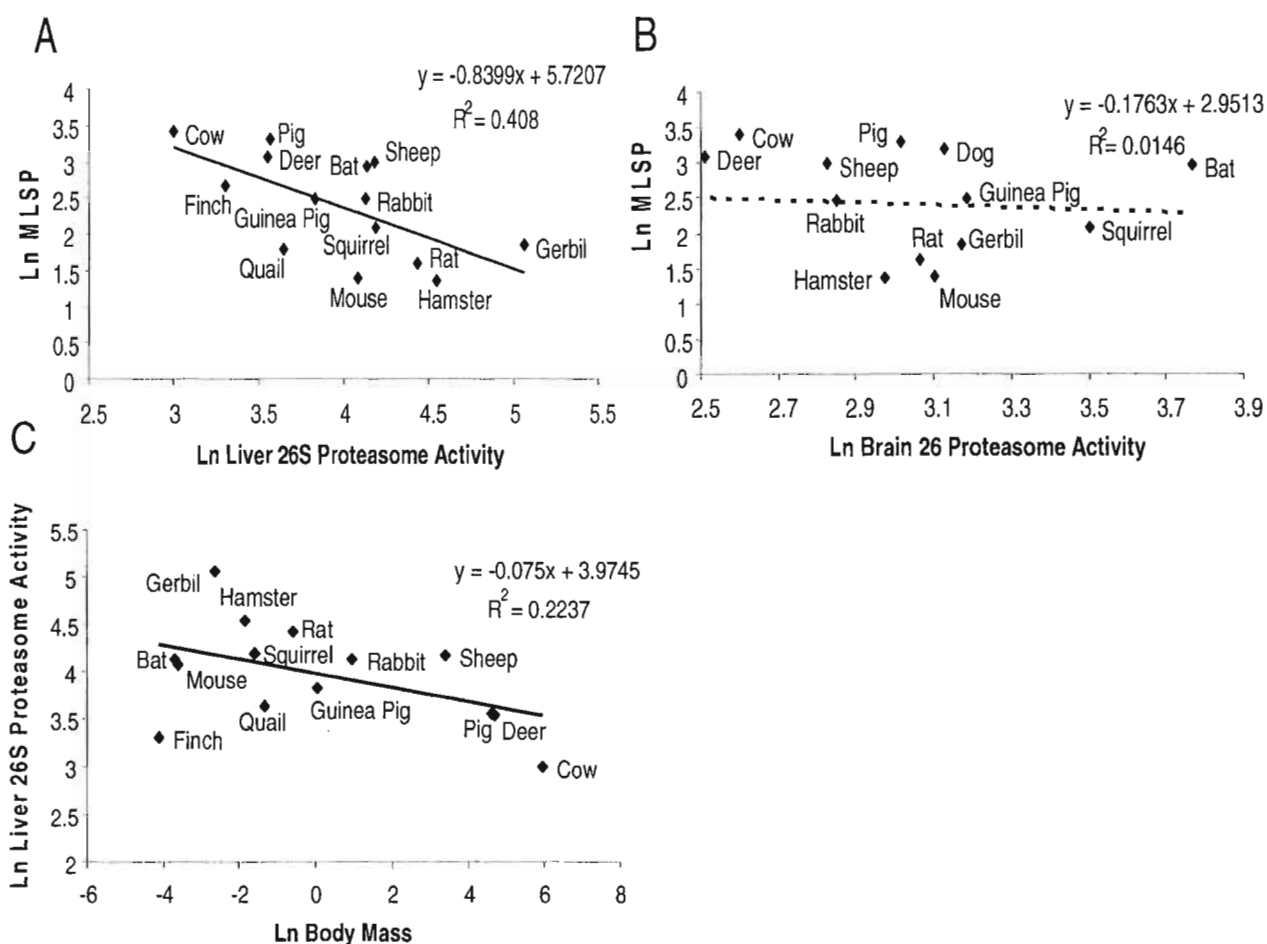
## 2.3 Results

Activity of the 20S proteasome was measured in liver, heart and brain tissue of 3-6 individuals from each of 13 mammalian and 2 avian species. The 20S proteasome activity data were natural logarithm (Ln) transformed and plotted against Ln-transformed MLSP data for each species (Fig. 2.1A-C). A correlational analysis revealed a significant inverse relationship between the two parameters in liver, but not heart or brain. To test whether the negative correlation between 20S proteasome activity in liver and MLSP (Fig. 2.1A) was actually driven by body mass, we plotted the residuals of the body mass versus MLSP correlation against the residuals of the body mass versus 20S proteasome activity (Fig. 2.1D). This analysis yielded no significant correlation, indicating the absence of a relationship between 20S proteasome activity and body mass. The direct correlation of liver 20S proteasome activity with body mass, which is significant ( $P < 0.05$ ) is shown in Fig. 2.1E (see also Table 2.3). Because basal metabolic rate is inversely correlated with body mass, we hypothesized that liver 20S proteasome activity may be influenced by mass-specific metabolic rate. This was addressed using mass-specific metabolic rate data from AnAge (Magalhaes et al. 2005).



**Figure 2.1.** Correlations between 20S proteasome activity and MLSP. (A) 20S proteasome activity is negatively correlated with MLSP in liver, but not in heart (B) or brain (C). Analysis of the residuals of 20S proteasome activity plotted against residual of MLSP (D) shows the absence of a statistically significant correlation. (E) Statistically significant correlation of liver 20S proteasome activity with body mass ( $p < 0.05$ ). All values are natural-log transformed and each data point represents the mean from 3-8 individuals of a given species.

The 20S proteasome plays a key role in the degradation of oxidatively damaged proteins that are not ubiquitin-tagged (Reinheckel et al. 1998; Davies et al. 2001). Conversely, ubiquitin-tagged proteins are degraded by the 26S proteasome (Jung et al. 2009). To estimate the capacity for degrading ubiquitin-tagged proteins, 26S proteasome activity was measured in liver and brain tissue samples, generating similar results to 20S proteasome activity. The activity of the 26S proteasome was negatively correlated ( $P < 0.05$ ) with MLSP in liver tissue (Fig 2.2A), but we observed no correlation between 26S proteasome activity and MLSP in brain tissue (Fig 2.2B). Again, analysis of the residuals of liver 26S proteasome activity revealed no correlation with MLSP or mass-specific metabolic rate, and these results were found also using FIC analysis (Table 2.2). Thus, liver 26S proteasome activity also correlates negatively with body mass (shown in Fig 2.2C, Table 2.3).

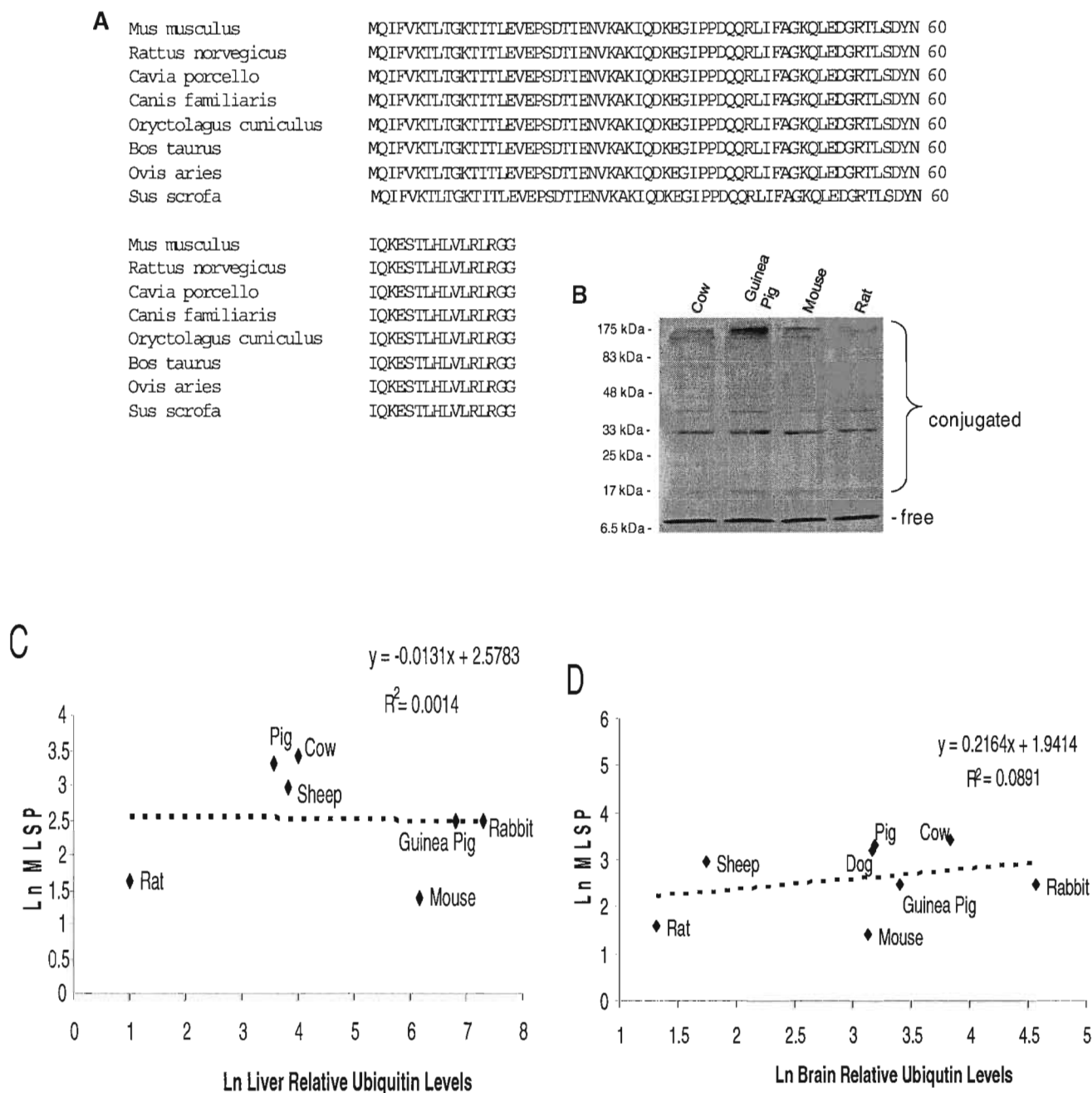


**Figure 2.2** Correlation between 26S proteasome activity and MLSP. 26S proteasome activity correlates negatively with MLSP ( $p < 0.025$ ) in liver (A) but not brain (B). The trend in (A) is driven by the correlation of liver 26S proteasome activity with body mass (C). All values are natural-log transformed and each data point represents the mean from 3-8 individuals of a given species.

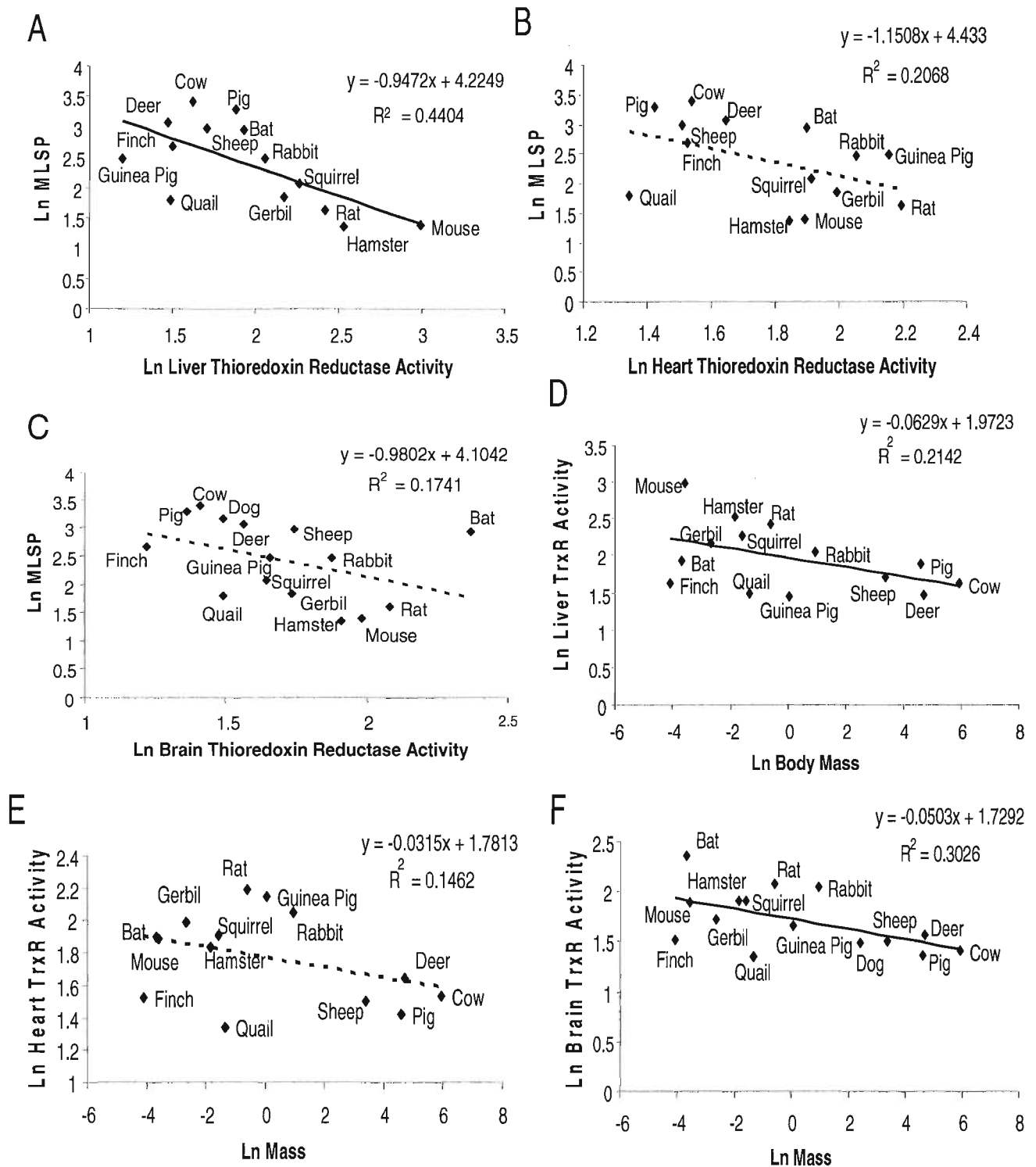
*In vivo* activity of the 26S proteasome will be affected by the concentration of its substrates (i.e. ubiquitinated proteins). Steady-state levels of protein ubiquitylation were measured in liver and brain tissues using quantitative immunodetection. Surprisingly, the amino acid sequence of ubiquitin is 100% conserved amongst eight species for which we could obtain complete sequence data (Fig. 2.3A). This allowed the

quantitative cross-species western quantification of ubiquitinated protein levels (Fig. 2.3B shows a representative blot). The total ubiquitin signal (bound and free) per lane was quantified and plotted against MLSP for these species (Fig. 2.3C-D). The analysis revealed no correlation between ubiquitinated protein levels and MLSP or body mass (data not shown) in either liver or brain tissue. The results were unaffected when measuring only protein-bound ubiquitin (data not shown).

TrxR activity appeared to be negatively correlated with MLSP in all three tissues; however, this reached statistical significance only in liver (Fig. 2.4A-C). Again, the liver relationship was driven by the negative correlation of TrxR with body mass (Fig. 2.4D, Table 2.2). Interestingly, while brain TrxR activity did not correlate significantly with MLSP (Fig. 2.4E) or with mass-specific metabolic rate (Table S1), the correlation of brain TrxR activity with body mass was significant (Fig. 2.4F, Table 2.3).

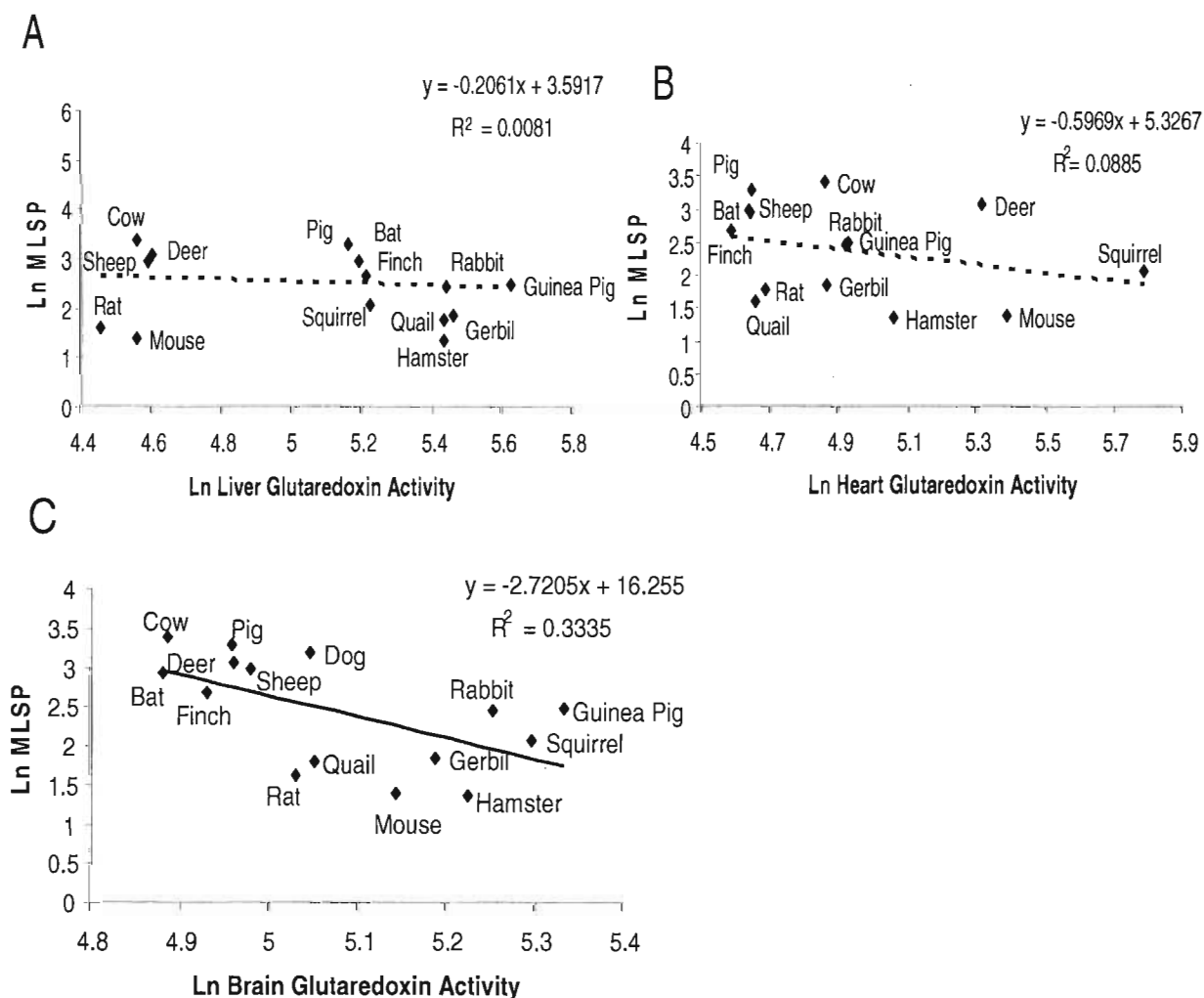


**Figure 2.3.** Ubiquitin protein levels do not correlate with MLSP. (A) The amino acid sequence of ubiquitin is conserved in the seven species analyzed. (B) A representative western detection of ubiquitin in electrophoresed rabbit liver homogenate proteins of four individuals. Sizes in kDa represent protein marker sizes. Lanes represent ubiquitin protein levels in liver of different species. (C,D) No correlation between extent of protein ubiquitylation and MLSP in (C) liver and (D) brain tissue. All values are natural-log transformed and each data point represents the mean from 3-8 individuals of a given species.



**Figure 2.4.** Correlation between thioredoxin reductase (TrxR) activity and MLSP or body mass. TrxR activity is negatively correlated with MLSP (A) in liver ( $p < 0.025$ ), but not in (B) heart or (C) brain tissue. (D-F) Correlation of TrxR activity with species' body mass. TrxR is negatively correlated with body mass in (D) liver ( $p < 0.05$ ), and (F) brain tissue, but not in (E) heart tissue. All values are natural-log transformed and each data point represents the mean from 3-8 individuals of a given species.

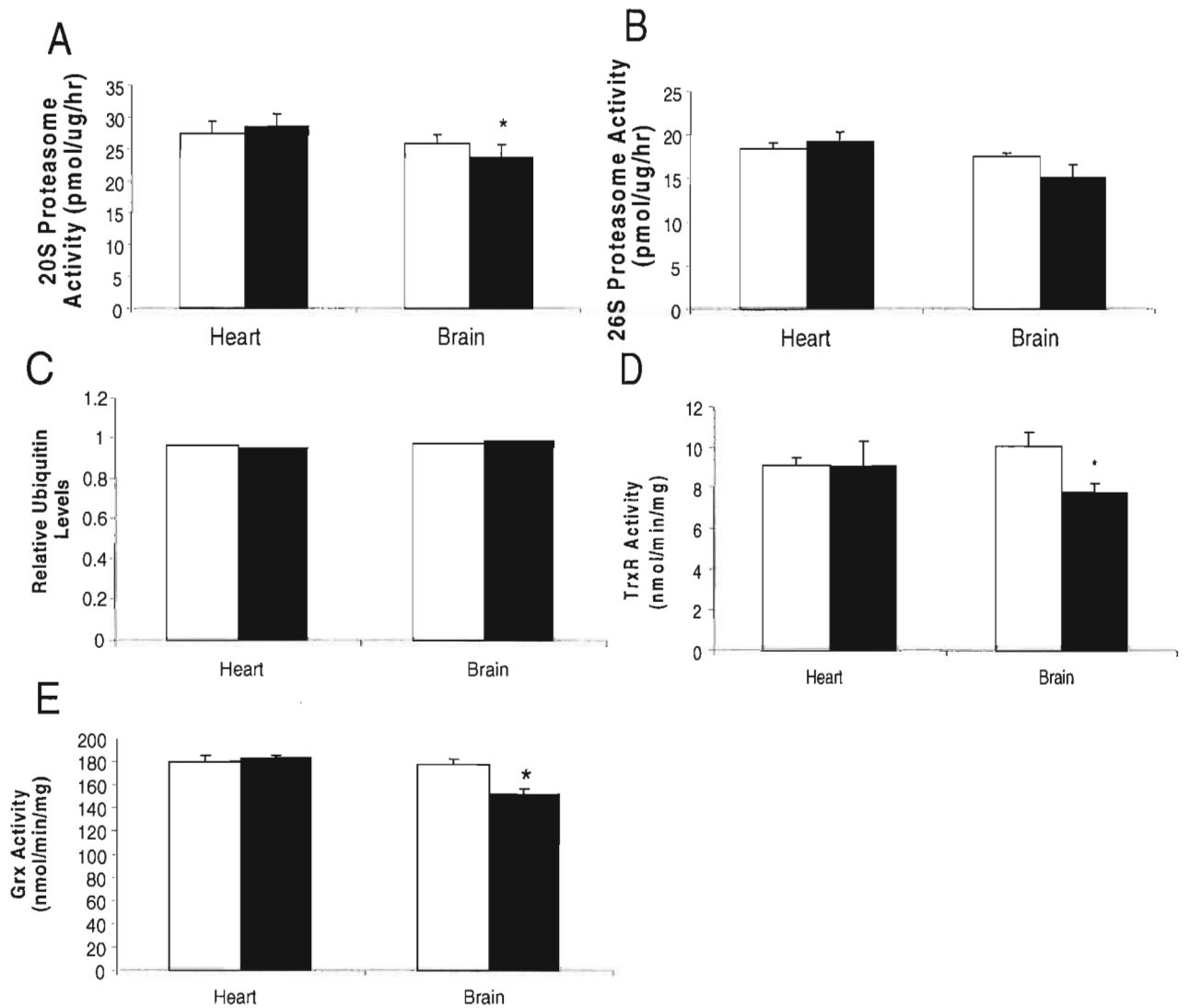
Grx activity was not correlated with either MLSP (Fig. 2.5A-B) or body mass (not shown) for either liver or heart. However, brain Grx activity was significantly negatively correlated with MLSP (Fig. 2.5C). In this instance, the correlation remained following residual analysis (Table 2.2), though was lost following FIC analysis (Table 2.2). Correlations between Grx activity and either species' body mass (Table 2.3) or mass specific metabolic rate (Table S1) were also not statistically significant.



**Figure 2.5.** Correlation between glutaredoxin (Grx) activity and MLSP. Grx activity in (A) liver and (B) heart do not correlate with MLSP. Brain Grx does correlate negatively with MLSP (C) ( $p < 0.025$ ). All values are natural-log transformed and each data point represents the mean from 3-8 individuals of a given species.



We measured the same protein repair and degradation activities in Snell normal and dwarf mice, an intra-specific model of increased longevity. There were no significant differences between normal and dwarf mice in 20S or 26S proteasome activities, TrxR or Grx in heart tissue. However, brain 20S proteasome activity was significantly lower in Snell dwarf, compared to normal, mice (Fig 2.6A). The same trend was observed for brain 26S proteasome activity, though this did not reach statistical significance. Protein ubiquitination levels did not differ between dwarf and normal heart or brain tissues. However, both TrxR and Grx activities were also lower in brain of Snell dwarf compared to normal mice (Fig. 2.6B-E).



**Figure 2.6.** Protein degradation and protein repair enzymes are not upregulated in long-lived Snell dwarf mice compared to normal controls. (A) 20S proteasome activity is significantly lower in Snell normal mice in brain tissue but no significance is found in heart tissue. (B) 26S proteasome activity and (C) ubiquitin protein levels are not different between Snell dwarf and Snell normal mice in brain tissue. (D) Thioredoxin reductase and (E) glutaredoxin activity levels are significantly higher in Snell normal mice compared to Snell dwarf mice in brain tissue but not in heart tissue. White bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means  $\pm$  SEM of duplicate measurements made in each of five individuals per genotype. ‘\*’ = significantly different ( $p < 0.05$ ) using a two-tailed t-test.

## 2.4 Discussion

The aim of this study was to test the hypothesis that longer-lived species or mouse strains have greater protein repair and/or recycling capacity. The hypothesis stems from the disposable soma theory of aging, which suggests that increases in energy invested in cellular maintenance will translate into increases in lifespan. This theory is supported by observations, in a wide variety of experimental models, of enhanced cellular stress resistance concomitant with greater longevity (reviewed in Robb et al. 2009). The 20S/26S proteasome appears to be a good candidate for positive selection by species evolving increased lifespan. It is required for the efficient removal of damaged proteins following oxidative stress (Divald and Powell 2006) and is stress-inducible (Grune et al. 1998). In *C. elegans*, fully functional 26S proteasome activity is required for normal stress resistance and lifespan (Ghazi et al. 2007). Its inhibition induces mitochondrial dysfunction, ROS overproduction and oxidative stress (Torres and Perez 2008). It is therefore interesting that we found no evidence of positive selection of either 20S or 26S proteasome activity during the evolution of longevity in vertebrate endotherms. Indeed, in this study longer-lived species generally possessed lower 20S/26S proteasome activities in liver, though this trend was driven by the negative correlation of proteasome activity with adult body mass. It was surprising that another negative correlate of body mass, mass-specific metabolic rate, was found also not to explain this negative correlation. While we do not have an explanation for the correlation between body mass and proteasome activity, we can in any case reject our hypothesis that the latter is related to MLSP.

The similar result from our intra-specific model of increased longevity, i.e. the Snell dwarf mouse, further strengthens our conclusion that the longevity of vertebrate endotherms is not associated with enhanced proteasome activity. This conclusion is consistent with two recent reports of proteasome activity in the context of mammalian species lifespan. Salmon et al. (2009) also reported significantly lower 20S proteasome activity in livers of two long-lived bat species, *Tadarida brasiliensis* and *Myotis velifer*, compared to mice. Interestingly, we did not find that liver 20S and 26S proteasome activities in our bat species, *Eptesicus fuscus*, were lower than those in mice. Indeed, liver 20S/26S proteasome activities were in the mid-range of all species values, whereas heart and brain proteasome activities were actually in the high end of the species range. There are numerous possible reasons for the differences between the present study and Salmon et al. (2009), including the use of different bat species. However, these contrasting results highlight the importance of using as many species as possible in such studies to avoid biasing conclusions.

One factor that will affect 26S proteasome activity *in vivo* will be the concentration of its substrates, i.e. ubiquitinated proteins. We measured the relative levels of protein ubiquitylation in liver and brain tissue from seven/eight species for which we were able to verify 100% conservation of the ubiquitin amino acid sequence. It is imperative to restrict this analysis to ubiquitin sequences for which 100% conservation can be verified as any between-species differences in sequence may affect the antigen-antibody interaction and thus render the quantification inaccurate. The absence of a correlation between ubiquitylation levels and MLSP, combined with the 26S proteasome results, indicate that the overall rate of protein degradation via the ubiquitylation route *in*

*vivo* is unlikely to be elevated in the longer-lived species. This suggests that degradative protein recycling via the proteasome does not contribute to any differences in protein homeostasis that may exist either between species or in the long-lived Snell dwarf mice.

Protein homeostasis in stressed cells may also be compromised by oxidation of redox-sensitive thiols, such as cysteine. Reversal of this oxidative damage is catalyzed by a number of enzymes using NADPH and glutathione to supply reducing equivalents. Here we tested, and reject, the hypothesis that longevity would be associated with greater activities of two such enzymes, TrxR and Grx, thus providing a greater ability to rapidly respond to oxidative damage to vulnerable cysteines. TrxR and Grx were either not correlated or negatively correlated with MLSP (depending upon tissue). In the latter instance, the relationship was found to be driven either by body mass (TrxR) or perhaps directly by MLSP (Grx). Combined with marginally lower TrxR and Grx activities in brain tissue from Snell dwarf, relative to normal mice, it would appear that maintenance of constitutively high protein repair activities does not contribute to longevity. These results are similar to those reported by Perez et al. (2009a), showing that neither glutaredoxin nor methionine sulfoxide reductase A levels are higher in naked mole rat liver compared to mouse (though this conclusion is limited by potential between-species differences in amino acid sequences in this study). Thus, longevity is not associated with enhanced protein repair activities in the major oxidative tissues.

Our results indicate that protein repair and degradative recycling activities are not elevated in longer-lived animals. Nonetheless, longevity is broadly associated with enhanced cellular stress resistance, including resistance to oxidative stressors (e.g. Kapahi et al., 1999; Ogburn et al. 2001; Robb et al. 2009; Miller et al. 2010). Perhaps

protein homeostasis is maintained by preventing the occurrence of damage, and research should focus on these mechanisms. However, some possible mechanisms of achieving this can also be eliminated. There is little evidence for greater antioxidant enzyme capacity in longer-lived species (reviewed in Perez et al. 2009b; Page et al. 2010a). Protein oxidative damage could also be prevented in longer-lived species by the systematic evolutionary replacement of cysteine residues with amino acids less susceptible to oxidative modification (as Moosmann and Behl (2008) demonstrated for mitochondrial DNA-encoded proteins); however, there is no evidence that this has occurred in nuclear DNA-encoded proteins that function either in the mitochondria or cytosol (Moosmann and Behl 2008). Similarly, proteins of naked mole rats actually have higher cysteine content than shorter-lived mice (Perez et al. 2009a). On the other hand, proteins in crude liver homogenates of some long-lived species, such as bats or naked mole rats, have been shown to resist urea-induced denaturation, which suggests some inherent stabilizing property (Perez et al. 2009a; Salmon et al. 2009). Perez et al. (2009a) report that this is not attributable to differences in heat shock protein-70 levels. While this between-species immunodetection approach requires validation for conservation of amino acid sequence, it is nonetheless in agreement with the observation that HSP70 levels are also lower in stress-resistant fibroblasts of long-lived Snell dwarf mice compared to those from normal littermates (Maynard and Miller 2006). A further study examining several different heat shock protein mRNA's in Snell dwarf mice found no coordinated elevation of HSPs in Snell dwarf tissues or fibroblasts (Swindell et al. 2009). These latter mRNA data may not reflect protein levels, as changes in gene transcript levels often do not parallel changes in corresponding protein levels, even for

transcriptionally regulated proteins (see for example Ideker et al. 2001; Le Naour et al. 2001). Investigation into HSP protein levels in this context is therefore probably still warranted. In addition, the possibility that specific post-translational protein modifications impart stability should be explored.

An important caveat to our study is that we measured total activities of proteins of interest in whole tissue homogenates, which precluded us from identifying between-species or between-strain differences that might be present within a specific cellular compartment. For example, aspects of mitochondrial function have been strongly implicated in aging and longevity (Barja and Herrero 2000; Boffoli et al. 1994; Navarro and Boveris 2007). All of the protein activities measured in this study have mitochondrial counterparts. Specific thioredoxin, TrxR and Grx isoforms localize to mitochondria in mammalian, and presumably avian, cells (see Page et al. 2010b). Similarly, mitochondria contain a Lon protease with similar stress-inducible protein degradation functions to the 20S/26S proteasome (Ngo and Davies 2009). These specific mitochondrial activities may be upregulated concomitantly with increased longevity, a possibility that should be explored in future studies.

In conclusion, we show that neither protein repair nor proteasomal degradation is positively correlated with longevity in either a multi-species or an intra-species comparison. These results support the idea that enhanced cellular stress resistance associated with longevity does not arise from increased ability to repair or remove stress-induced protein damage.

Table 2.2. Statistical analysis of linear regressions: enzyme activity as a function of lifespan.

| Enzyme Activity             | Correlation ( $R^2$ ) | Slope   | P-Value |
|-----------------------------|-----------------------|---------|---------|
| <b>Liver 20S proteasome</b> |                       |         |         |
| Residual                    | 0.2087                | -0.4087 | > 0.05  |
| Residual + FIC              | 0.00003               | 0.0044  | > 0.05  |
| <b>Heart 20S proteasome</b> |                       |         |         |
| Residual                    | 0.0023                | 0.0539  | > 0.05  |
| Residual + FIC              | 0.0143                | 0.1164  | > 0.05  |
| <b>Brain 20S proteasome</b> |                       |         |         |
| Residual                    | 0.0072                | -0.0809 | > 0.05  |
| Residual + FIC              | 0.0013                | -0.0353 | > 0.05  |
| <b>Liver 26S proteasome</b> |                       |         |         |
| Residual                    | 0.0004                | -0.0227 | > 0.05  |
| Residual + FIC              | 0.0125                | -0.0984 | > 0.05  |
| <b>Brain 26S proteasome</b> |                       |         |         |
| Residual                    | 0.0046                | -0.0728 | > 0.05  |
| Residual + FIC              | 0.0493                | -0.3133 | > 0.05  |
| <b>Liver TrxR</b>           |                       |         |         |
| Residual                    | 0.1306                | -0.5568 | > 0.05  |
| Residual + FIC              | 0.0095                | -0.1635 | > 0.05  |
| <b>Heart TrxR</b>           |                       |         |         |
| Residual                    | 0.0834                | -0.5849 | > 0.05  |
| Residual + FIC              | 0.0003                | 0.0386  | > 0.05  |
| <b>Brain TrxR</b>           |                       |         |         |
| Residual                    | 0.0016                | -0.0823 | > 0.05  |
| Residual + FIC              | 0.0385                | -0.3822 | > 0.05  |
| <b>Liver Grx</b>            |                       |         |         |
| Residual                    | 0.0151                | 0.1675  | > 0.05  |
| Residual + FIC              | 0.0012                | -0.0391 | > 0.05  |
| <b>Heart Grx</b>            |                       |         |         |
| Residual                    | 0.1194                | -0.5137 | > 0.05  |
| Residual + FIC              | 0.0299                | -0.213  | > 0.05  |
| <b>Brain Grx</b>            |                       |         |         |
| Residual                    | 0.2752                | -1.8917 | < 0.05  |
| Residual + FIC              | 0.0353                | -1.0398 | > 0.05  |



Table 2.3. Statistical analysis of enzyme activities as correlates of body mass

| Enzyme Activity                  | Correlation ( $R^2$ ) | Slope   | P-Value |
|----------------------------------|-----------------------|---------|---------|
| Liver 20S<br>Proteasome Activity | 0.2548                | -0.1008 | <0.05   |
| Heart 20S<br>Proteasome Activity | 0.1520                | -0.0583 | >0.05   |
| Brain 20S<br>Proteasome Activity | 0.0866                | -0.0501 | >0.1    |
| Liver 26S<br>Proteasome Activity | 0.2237                | -0.075  | <0.05   |
| Brain 26S<br>Proteasome Activity | 0.0178                | -0.0196 | >0.1    |
| Liver TrxR Activity              | 0.2142                | -0.0629 | <0.05   |
| Heart TrxR Activity              | 0.1462                | -0.0315 | >0.05   |
| Brain TrxR Activity              | 0.3026                | -0.0503 | <0.05   |
| Liver Grx Activity               | 0.1571                | -0.0489 | <0.1    |
| Heart Grx Activity               | 0.0040                | -0.0066 | >0.1    |
| Brain Grx Activity               | 0.0984                | -0.0143 | >0.1    |

## **Chapter 3: Higher levels of heat shock proteins in longer-lived mammals and birds**

### **Hypothesis**

Molecular chaperones play a role in conferring longevity in longer-lived species or strains.

### **Objective**

The primary objective of this project was to determine if protein refolding of protein through molecular chaperones play a role in animal longevity in an inter-specific and intra-specific context.

### **Publication**

**Salway KD, Gallagher EJ, Page MM, Stuart JA.** Higher levels of heat shock proteins in longer-lived mammals and birds. *Mech Ageing Dev. In press*

### **Contributions**

I performed all experiments, phylogenetic and statistical analysis and chapter write-up.

### 3.1 Introduction

Vertebrates are a diverse clade with a wide range of maximum lifespans (MLSP) from several years to over a century. Yet, the phenotype of aging appears remarkably similar amongst these species, suggesting it may stem from common cellular and molecular mechanisms. Therefore, it may be possible to identify specific molecular traits that have co-evolved with longevity and indeed may facilitate it. We currently have a limited understanding of what these traits are and their determination remains a major goal. Previous studies have shown that the cells of longer-lived species are characterized by greater resistance to exogenous stressors (Kapahi, 1999; Kirkwood et al. 2000; Ogburn et al. 2001; Miller et al. 2010).

Protein homeostasis is a critical component of cellular maintenance and stress resistance. Oxidative stress causes damage that alters protein structure and function, and can promote aggregation, as has been well characterized in several neurodegenerative diseases (Reviewed in Ali et al., 2010). The importance of maintaining proteins in their correct conformation is illustrated by the robust unfolded protein responses (UPR) that manifest in various cellular compartments, including cytosol, mitochondria and endoplasmic reticulum (ER). The core effectors of the UPR in any compartment are heat shock proteins (Hsps), which range in size and activity, but function in a broad variety of protein folding, refolding and chaperoning activities (reviewed in Bukau et al., 2006). The importance of Hsps in aging and longevity has been illustrated by experiments in *Caenorhabditis elegans* and *Drosophila* species. For example, overexpression of hsp-6 (Yokoyama et al., 2002) and hsp-16 (Walker and Lithgow, 2003) genes extend lifespan in *C. elegans*, and heat shock factor 1 (HSF-1), which transcriptionally regulates the heat

shock response, is required for lifespan extension in *daf-2 C. elegans* mutants (Hsu et al., 2003). Overexpression of Hsps is also associated with lifespan extension in *D. melanogaster* (Morrow et al., 2010). In mice, however, the data are much more equivocal. Overexpression of Hsp70 and HSF1 have been associated with increased longevity in disease models (e.g. Gifondorwa et al., 2007; Steele et al., 2008; Hayashida et al., 2010). However, overexpression of Hsp70 in healthy mice does not extend lifespan (Vanhooren et al 2008). Also, tissue Hsp mRNA levels are not globally upregulated in long-lived dwarf or growth hormone/insulin-like growth factor-1 signalling pathway deficient mice (reviewed in Swindell 2009). Thus, there is a need to further investigate the relationship between tissue Hsp levels and longevity in mammals.

The cytosol, ER and mitochondria are distinct subcellular compartments within which protein folding states must be maintained independently. Hsp70 is one of the main cytoplasmic Hsps. In the ER, glucose-regulated protein 78 (GRP78) and 94 (GRP94) are key mediators of the UPR and thus major contributors to the ER stress response. Mitochondria also contain chaperones that support proper folding of newly translocated proteins and of those synthesized within the mitochondrial matrix. Hsp60 resides predominantly in the mitochondria, and has been shown to be selectively upregulated during the mitochondrial UPR (Corydon et al., 2005; Lee, 2005; Zhao et al., 2002).

In this study, we tested the hypothesis that the evolution of longevity in vertebrate endotherm species has selected for increasing protein folding capacities to promote protein homeostasis concomitantly with longer lifespans. We used antibodies raised to conserved epitopes to measure levels of mitochondrial Hsp60, ER GRP78 and GRP94, and cytosolic Hsp70 in liver, heart and brain of young adults representing 13 vertebrate

endotherm species ranging in maximum lifespan (MLSP) from 3 to 30 years (Table 1). We used residual analysis to differentiate associations with body mass from those with MLSP, and Felsenstein's independent contrasts (Felsenstein 1985) to address problems associated with non-independence of data points (i.e. species) due to shared evolutionary history.

Finally, we also measured Hsp60, Hsp70 and GRP78 in heart and brain from young adult Snell dwarf mice, a well-characterized intra-specific model of longevity, to examine whether the reported absence of uniform Hsp mRNA upregulation in long-lived dwarves (Swindell et al., 2009) is paralleled by protein levels.

## **3.2 Materials and Methods**

### ***3.2.1 Materials***

Chemicals were purchased from Bioshop (Burlington, ON, Canada) and Sigma Aldrich (Oakville, ON, Canada; including Fluka and Caledon). BioRad protein dye was purchased from BioRad Laboratories (Hercules, California, USA). Anti-Hsp60, anti-GRP94, anti-GRP78 and anti-Hsp70 primary antibodies were purchased from Abcam (Cambridge, Massachusetts, USA). Anti-rabbit and anti-goat secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, US). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA). Memcode reversible protein stain was purchased from Thermo Scientific (Rockford, Illinois, USA).

### ***3.2.2 Animals***

Between 3 and 6 individuals of each of 12 mammalian and 1 avian species were used in this study (Table 1) as in Chapter 2.

### ***3.2.3 Tissue sampling***

Tissues were collected as in Chapter 2.

### ***3.2.4 Preparation of Tissue Homogenates***

Tissue homogenates were prepared as in Chapter 2.

### ***3.2.5 Quantification of Heat Shock Protein Levels***

Brain, heart and liver homogenates (20µg) were separated by SDS-PAGE and electrotransferred to a PDVF membrane. Even protein transfer was verified using Memcode reversible protein stain prior to immunoblotting. The membranes were then incubated in a blocking solution, consisting of 5% skim milk in 1x PBS, for 45 min at room temperature, and then probed with anti-Hsp60 (1:2500), anti-GRP78 (1:400), anti-Hsp70 (1:100) or anti-GRP94 (1:100 for liver; 1:250 for heart and brain) primary antibodies in 5% skim milk-1x PBS-T overnight at 4°C. After several washes, membranes were then probed with anti-rabbit secondary antibody for 1 hour at room temperature, then washed and visualized using an Odyssey infrared visual system (LI-COR Biosciences). Protein level quantification was performed using Odyssey Imaging Software, Version 1.0. For each gel, band intensities were normalized to a rat sample that served as an internal standard. All measurements were made in duplicate and repeated for 3-8 individual animals, with loading position being randomly varied to minimize spurious effects due to uneven transfer. In addition, we performed parallel analyses where uneven protein loading/transfer were controlled for by summing the total

Memcode stained protein signal for each lane and standardizing Hsp/GRP levels to this value. These two approaches gave virtually identical trends.

### ***3.2.6 Statistical Analyses***

Statistical analyses were performed as in Chapter 2.

## **3.3 Results**

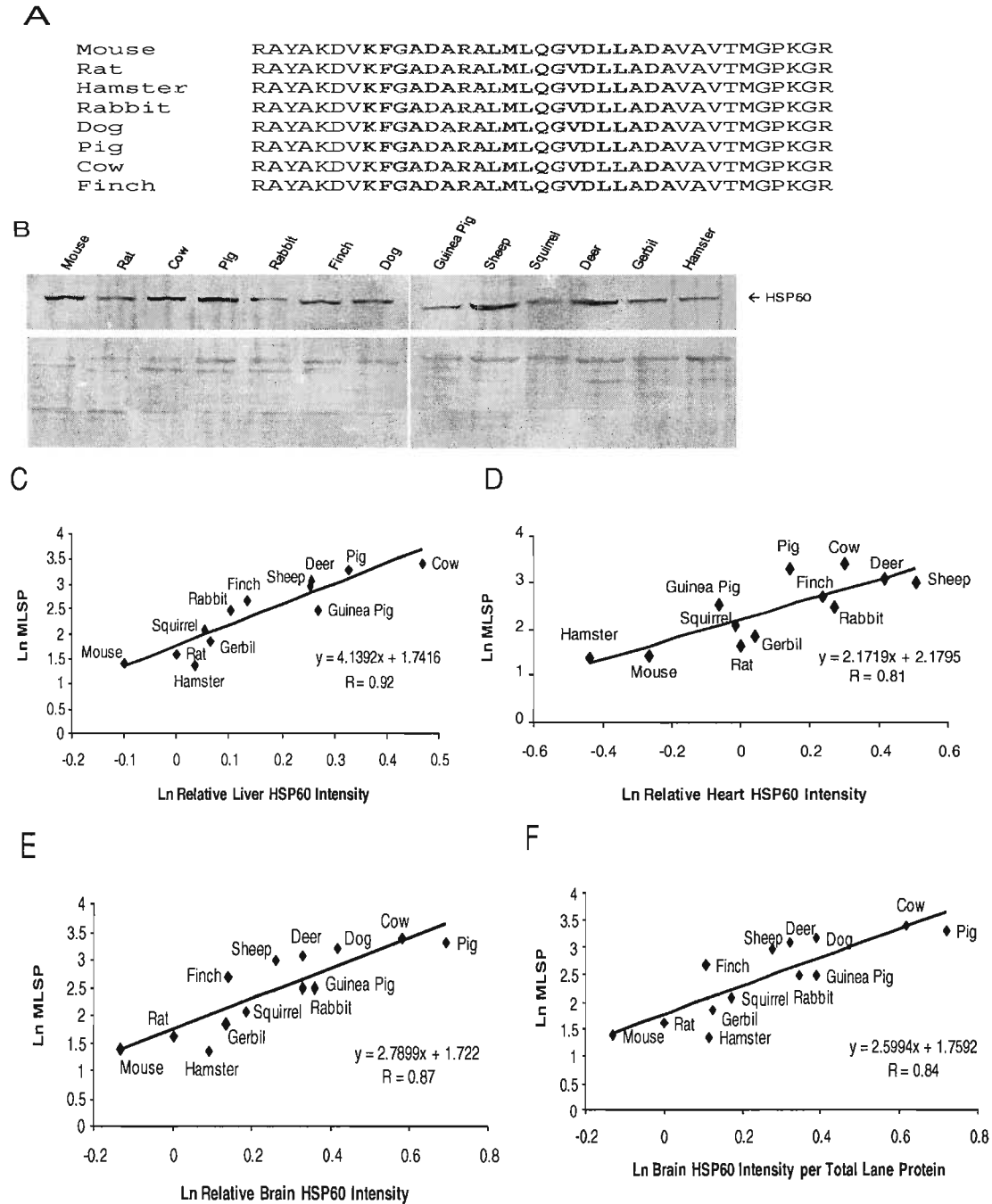
Typically, quantitative immunoblotting of Hsp protein levels in tissues of different species requires identifying regions within the amino acid sequence of an individual Hsp that is 100% conserved amongst the species included in the study. Fortunately, the amino acid sequences of several Hsps are exceptionally highly conserved amongst vertebrates. However, while full genome sequence data are available for many of the species used, we were not able to find sequence data for each Hsp for all species. For example, we found Hsp60 sequence data for eight species, including zebra finch (Fig. 3.1A), and reasoned that a region 100% conserved across these relatively phylogenetically divergent species was likely to be conserved also in the other five. A similar logic was applied when identifying suitable regions for GRP78 and GRP94, with available amino acid sequences and alignments across species shown in Figs. 3.2A and 3.3A. In each case, a commercial antibody was found that had been raised to an epitope within the region shown to be 100% conserved in all species for which sequence data was available. In the case of GRP78 (also called Hsp70-5 or Bip), it was necessary, in addition to the above condition, to select a sequence falling outside of regions that are highly conserved amongst different Hsp70 isoforms (see Daugaard et al., 2007). We selected a region near the N-terminus of GRP78 (Fig. 2) that, while fully conserved

across nine species for which sequence data could be found, is substantially divergent between Hsp70 isoforms. The one exception to this approach was for Hsp70. In this instance, we did not find an antibody to a region that was fully conserved amongst the species included in this study while also being substantially divergent between Hsp70 isoforms. Instead, we probed a region near the C-terminus of Hsp70 using an antibody that was designed to detect primarily Hsp70-1a and Hsp70-1b but may also bind to Hsp70-2, Hsp70-6 or Hsc, which have up to 70% sequence identity in this region. Therefore, interpretation of our 'Hsp70' measurements must take this into account. For each Hsp, an alignment of available sequences and the epitope to which the antibody used had been raised are indicated (Figs. 1-4).

Hsp60 levels in liver, heart and brain were determined in 12-13 species (not all tissues were available for each species). In each case, band intensity was normalized to the rat value for that particular tissue (Table 3.1). A correlational analysis revealed highly significant positive correlations ( $p < 0.05$ ) in liver, heart and brain tissues between MLSP and Hsp60 protein levels (Fig3.1B-D). In vertebrate endotherms, MLSP is highly correlated with body mass, and some relationships that appear to be with MLSP are actually driven by body mass. Therefore, to test whether a given correlation was actually driven by body mass, we plotted the residuals of the body mass versus Hsp60 protein levels against the residuals of body mass and MLSP. This analyses also yielded significant positive correlations ( $p < 0.05$ ) between MLSP and Hsp60 levels for each tissue (Table 3.2). As the data points, representing species, are not independent owing to their shared phylogenetic history, a FIC analysis was also performed to account for this limitation. The positive correlations between Hsp60 levels and MLSP remained



significant in each case ( $p < 0.05$ ; Table 3.2). In addition, to control for the possibility that unequal loading/transfer of sample proteins influenced the observed trends, we repeated the analyses standardizing Hsp signals to the total Memcode stained protein signal in each lane. This analysis yielded essentially identical trends for Hsp60 (Fig. 3.1F), GRP78 (Fig. 3.2F), GRP94 (Fig. 3.3F) and Hsp70 (Fig. 3.4F) for each tissue (only brain tissue results shown).



**Figure 3.1.** Correlations between tissue Hsp60 levels and species MLSP. A) Aligned HSP60 partial amino acid sequences showing region in which amino acid sequence identity is 100% and epitope (in red) to which antibody was raised. B) Representative western blot of Hsp60, and corresponding Memcode stained membrane, in brain tissue of 13 species. Hsp60 protein levels were positively correlated ( $p < 0.05$ ) with MLSP in C) Liver, D) Heart, and E) Brain. In (F) brain Hsp60 protein levels standardized to total lane Memcode signal are plotted against MLSP. This method of analysis produces essentially identical results. All values are natural-log transformed and each data point represents the mean of duplicate measurements made in 3-8 individuals of each species.

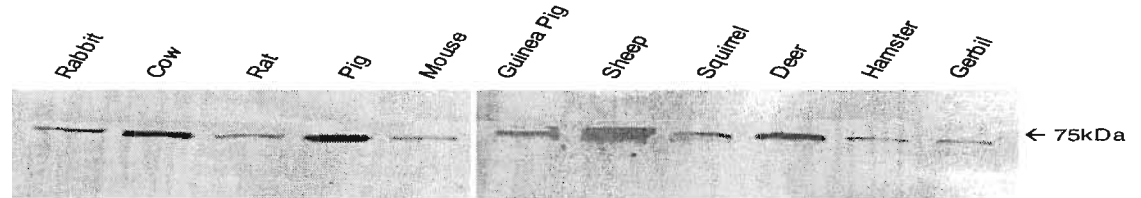
The levels of two ER Hsps, GRP78 and GRP94, were measured using the same basic approach. GRP78 was strongly correlated ( $p < 0.05$ ) with MLSP in all three tissues (Fig. 3.2). Analysis of residuals and application of FIC, as above, showed significant positive correlations ( $P < 0.05$ ) of GRP78 with MLSP remained for each tissue. Identical results were obtained for GRP94, which was correlated with MLSP in liver and heart ( $p < 0.05$ ; Fig. 3.3; Table 3.2), but the correlation in brain was significantly weakened ( $p < 0.1$ ) following analysis of residuals and correction using FIC.

Hsp70 levels were quantified using the same approach as above. Similarly, correlational analysis revealed significant positive correlations ( $p < 0.05$ ) in each of the three tissues between MLSP and Hsp70 protein levels (Fig. 3.4). These correlations remained following residual analysis and FIC analysis ( $p < 0.05$ ; Table 3.2).

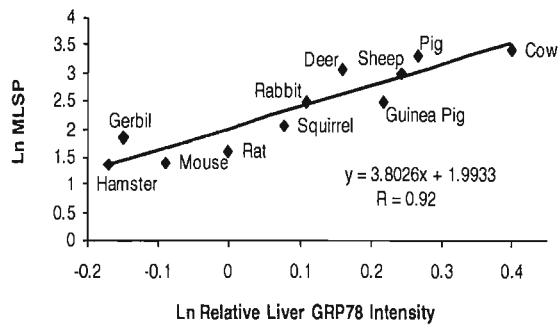
**A**

|          |                                     |
|----------|-------------------------------------|
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| Rat      | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Hamster  | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Rabbit   | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Squirrel | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Pig      | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Dog      | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |
| Cow      | EEEDKKEDVGTVVGIDLGTTYSVGVFKNGRVEI I |

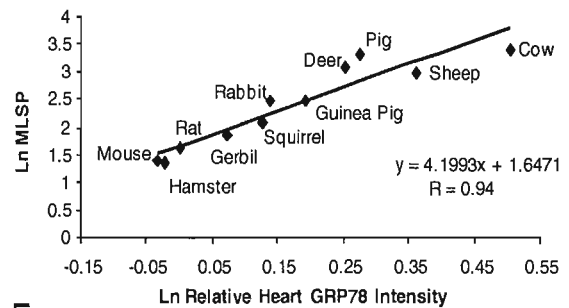
**B**



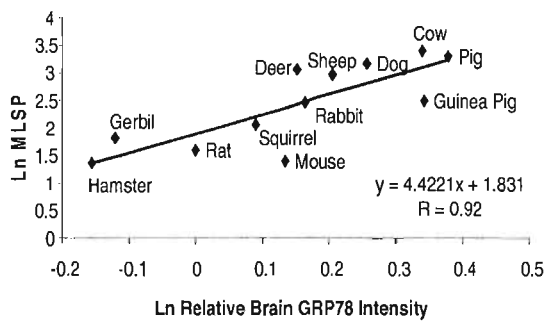
**C**



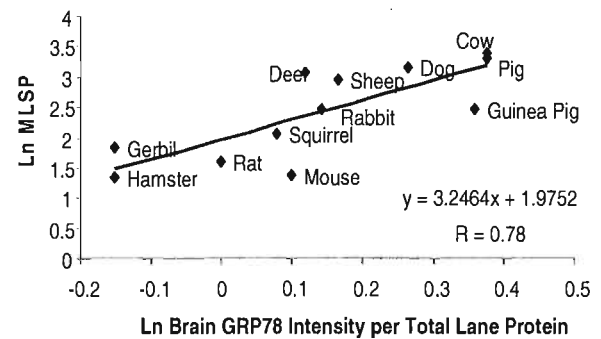
**D**



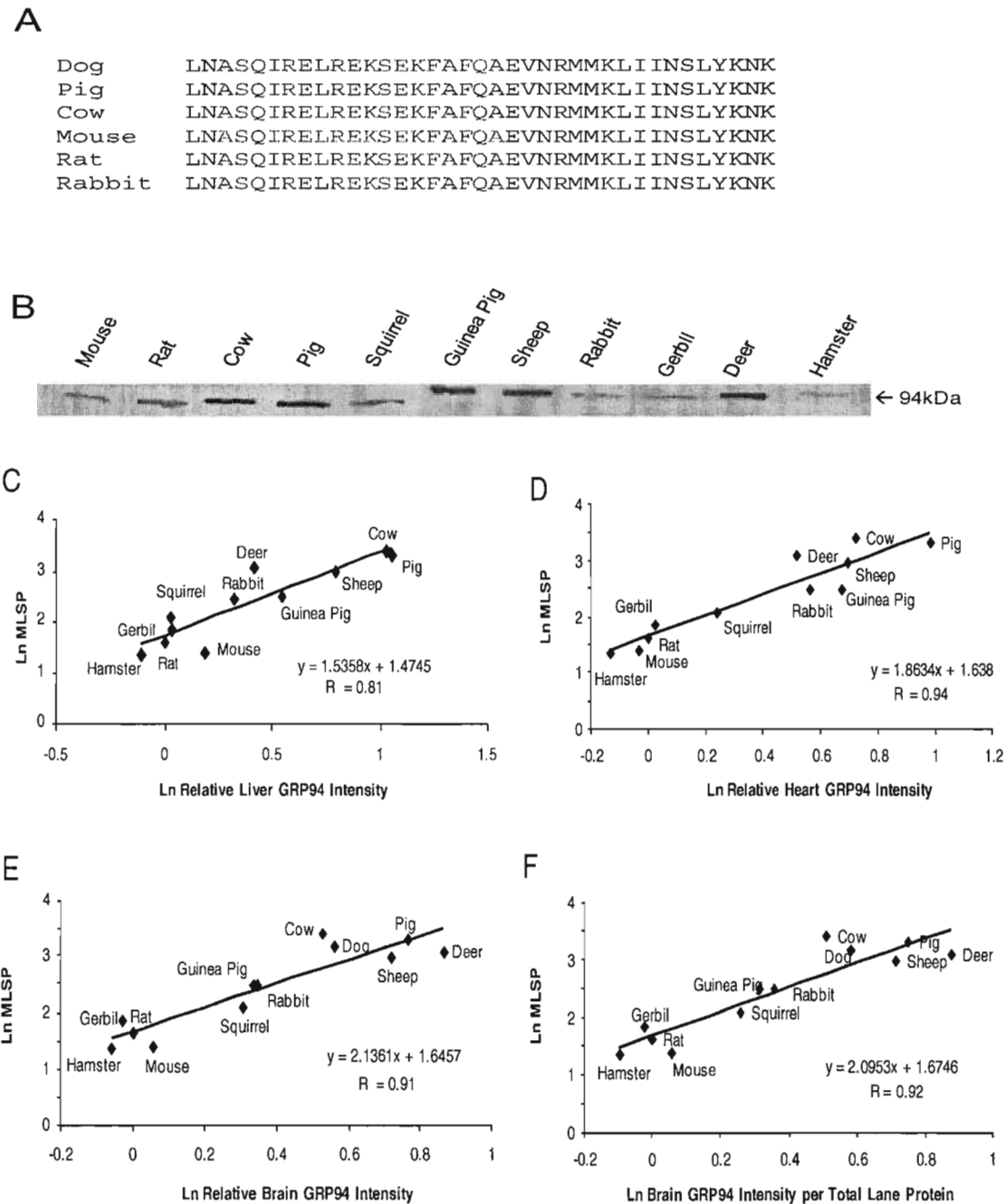
**E**



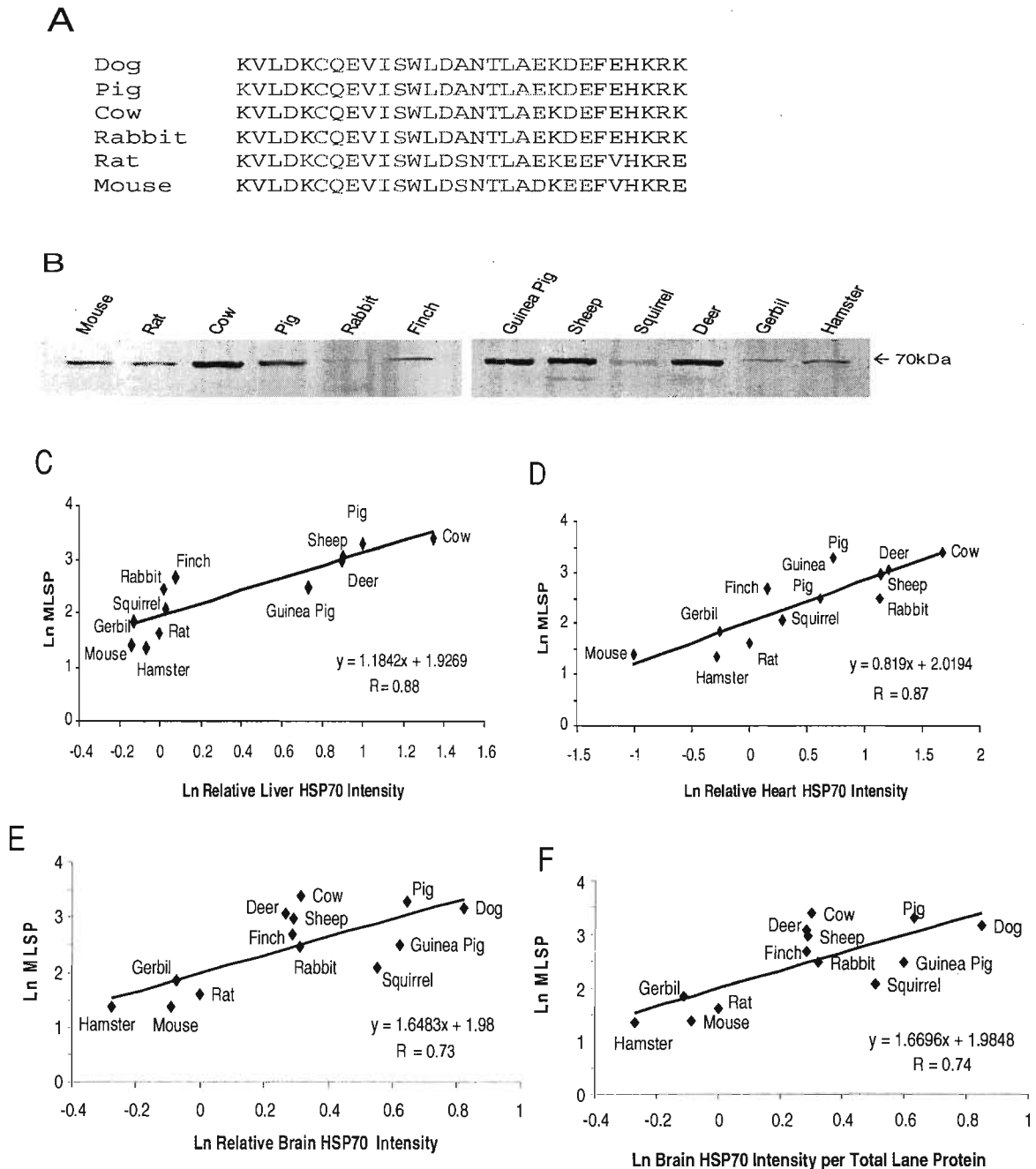
**F**



**Figure 3.2.** Correlation between GRP78 levels and species MLSP. A) Aligned GRP78 partial amino acid sequences showing region in which amino acid sequence identity is 100% and epitope (in red) to which antibody was raised. B) Representative western blot of GRP78 in heart. GRP78 protein levels were positively correlated ( $p < 0.05$ ) with MLSP in C) Liver, D) Heart, and E) Brain. In (F) brain GRP78 protein levels standardized to total lane Memcode signal are plotted against MLSP. This method of analysis produces essentially identical results. All values are natural-log transformed and each data point represents the mean from duplicate measurement from 3-8 individuals of a given species.

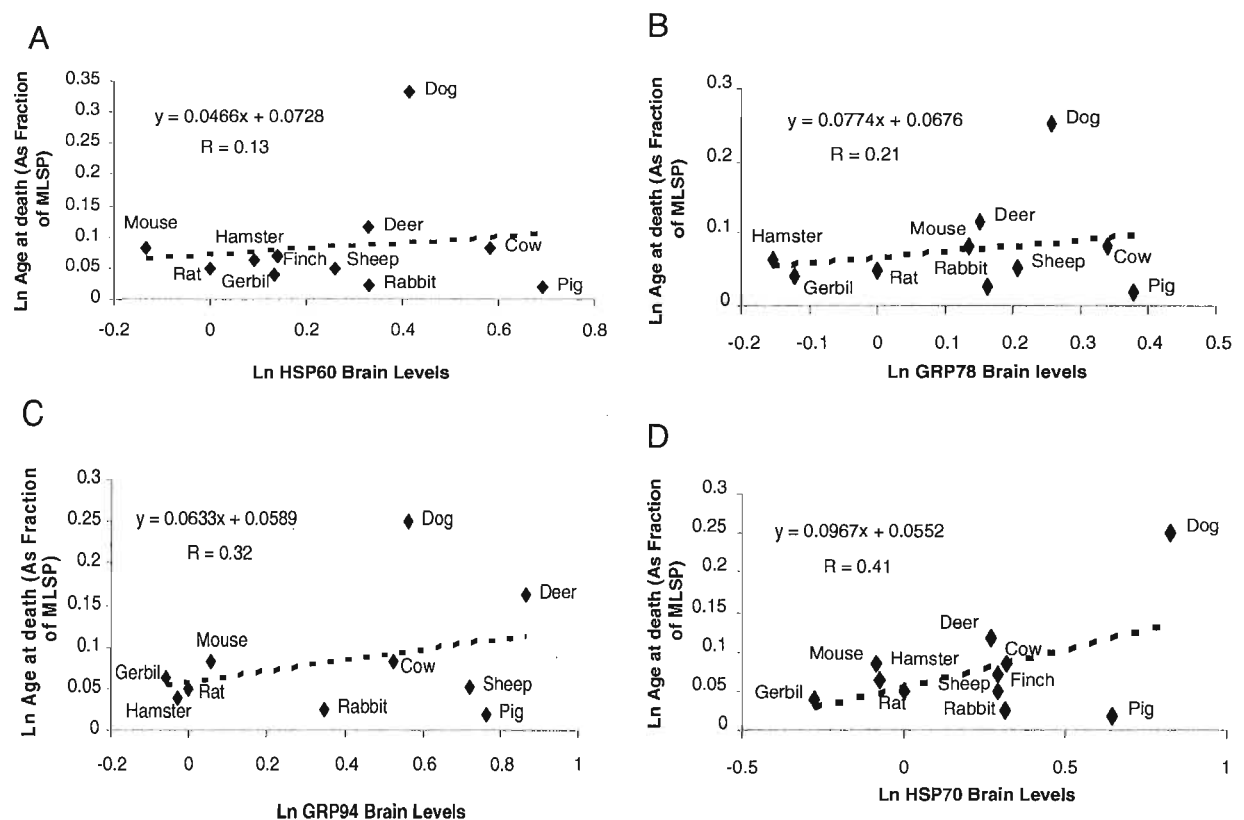


**Figure 3.3.** Correlation between GRP94 levels and species MLSP. A) Aligned GRP94 partial amino acid sequences showing region in which amino acid sequence identity is 100% and epitope (in red) to which antibody was raised. B) Representative western blot of GRP94 in liver. GRP94 protein levels are positively correlated ( $p < 0.05$ ) with MLSP in C) Liver, D) Heart and E) Brain. In (F) brain GRP94 protein levels standardized to total lane Memcode signal are plotted against MLSP. This method of analysis produces essentially identical results. All values are natural-log transformed and each data point represents the mean from duplicate measurement from 3-8 individuals of a given species.



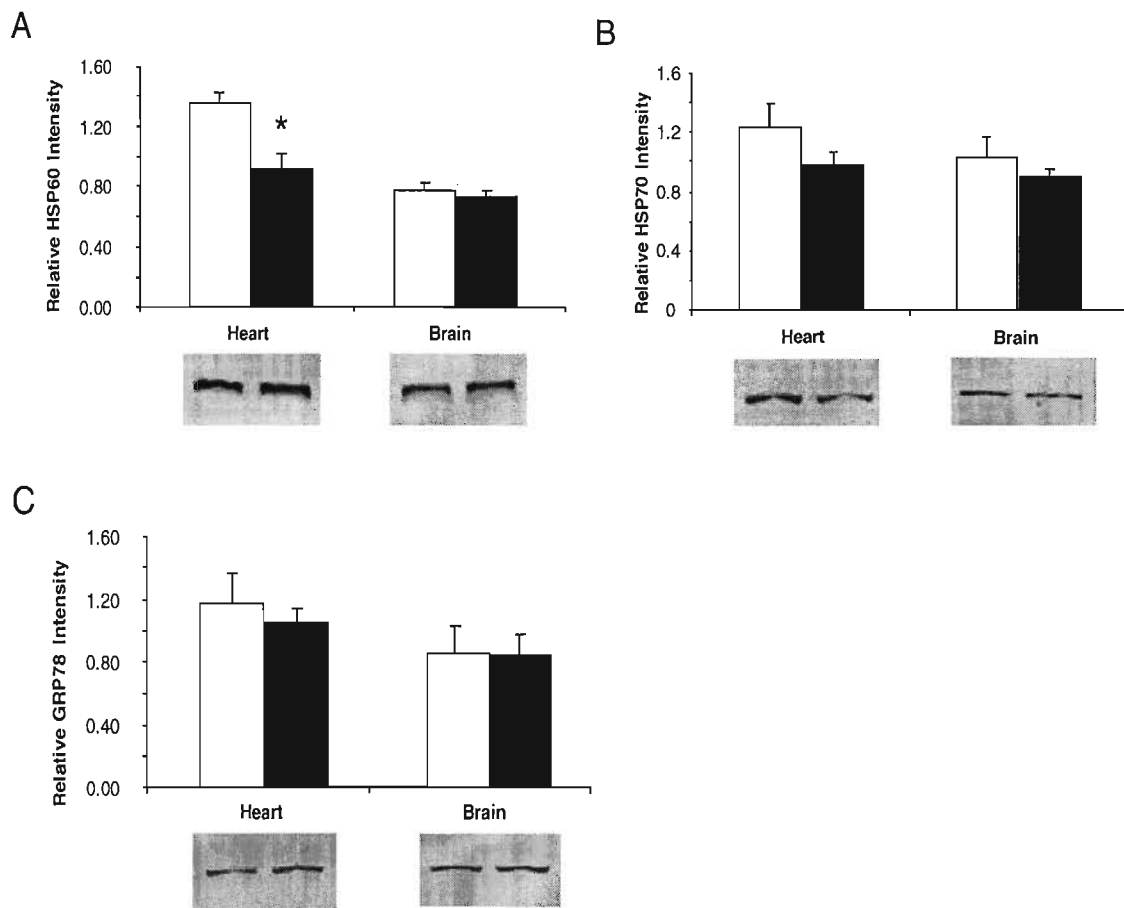
**Figure 3.4.** Correlation between tissue Hsp70 levels and MLSP. A) Aligned HSP70 partial amino acid sequences showing region in which amino acid sequence identity is highly conserved, and the epitope (in red) to which antibody was raised. B) Representative western blot of Hsp70 in heart. Hsp70 protein levels were positively correlated ( $p < 0.05$ ) with MLSP in C) Liver, D) Heart and E) Brain. In (F) brain Hsp70 protein levels standardized to total lane Memcode signal are plotted against MLSP. This method of analysis produces essentially identical results. All values are natural-log transformed and each data point represents the mean from duplicate measurement from 3-8 individuals of a given species.

Hsp expression is affected by a variety of variables, including age (typically Hsp levels and/or inducibility are reduced with age) and plasma IGF-1 levels. We therefore determined whether the age of individuals at the time of euthanization influenced measured Hsp levels. However, there were no significant correlations between this age (as a proportion of MLSP) and the level of any Hsp. This is shown only for brain tissue (Fig. 3.5), but a similar absence of significant correlations was found in heart and liver tissues. Similarly, using plasma IGF-1 data from Stuart and Page (2010) we determined that no correlations exist between plasma IGF-1 and the levels of any Hsp or GRP (not shown).



**Figure 3.5.** No correlation between HSP levels and animal age at death (as a fraction of MLSP). A) Hsp60, B) GRP78, C) GRP94 and D) Hsp70 protein levels did not correlate with animal age as a fraction of MLSP in brain tissue. All values are natural-log transformed and each data point represents the mean from duplicate measurements made on samples from 3-8 individuals of a given species.

We measured Hsp60, Hsp70 and GRP78 in heart and brain tissue from young adult Snell normal and dwarf mice, an intra-specific model of extended longevity. No significant differences between genotypes in Hsp70 or GRP78 protein levels were found in heart tissue, but Hsp60 was significantly lower in Snell dwarf mice compared to normal littermates ( $P < 0.05$ ; Fig. 3.6). In brain tissue, there were no significant differences in the levels of any of the three Hsps between Snell normal and dwarf mice (Fig. 3.6).



**Figure 3.6.** Heat shock proteins are not upregulated in long-lived Snell dwarf mice compared to normal littermates. Relative A) Hsp60; B) Hsp70; C) GRP78 protein levels and representative western blots in heart and brain tissue of young adult Snell dwarf mice and normal littermates. White bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means  $\pm$  SEM of duplicate measurements made on tissue samples from each of five individuals per genotype. '\*' = significantly different from normal mice ( $p < 0.05$ ) using a two-tailed t-test.



### 3.4 Discussion

Our data indicate that elevated Hsp expression in heart, brain and liver tissues are characteristic of longer-lived species. Combined with published studies showing the cause and effect relationship between Hsp expression and longevity in invertebrates, this suggests that Hsps are pro-longevity across metazoan phyla. While we measured only a small representative sample of Hsps, which number in the dozens in vertebrates (Chen et al., 2006; Daugaard et al., 2007; Calderwood et al., 2009), it is possible that other Hsps are similarly correlated with lifespan.

There is surprisingly little information regarding the effects on lifespan of Hsp overexpression in mammals, with reported data largely confined to experiments in which Hsp overexpression has successfully rescued premature aging and death in murine models of disease (e.g. McArdle et al., 2004; McLean et al., 2002). However, Vanhooren et al (2008) showed that transgenic overexpression of Hsp70 in mice actually shortened lifespan, while increasing tumour incidence. Thus, global Hsp70 overexpression, perhaps via its anti-apoptotic capacity in highly mitotic cancer cells, may negate the value of its protective function by promoting tumorigenesis. It would be interesting to know which cell types contribute to the elevated Hsp levels in tissues measured in the longer-lived species studied here. It seems reasonable that Hsp levels might be differentially expressed in different cell types. The abundant highly oxidative and highly differentiated cell types that comprise tissues like brain and heart (egs. myocytes, neurons) rather than actively mitotic cells are likely to be the primary contributors to the signals measured using our approach. However, a quantitative immunohistochemistry approach might provide insight into whether patterns of expression are unique to different cell types.

In addition to the potential for tumorigenesis, the transgenic manipulation of Hsp expression in mice may be complicated by the fact that these proteins play a variety of critical roles in development that could be negatively affected by lifelong systemic over- or under-expression. This is also evident in Hsp70 overexpressing mice (Vanhoories et al 2008), in which reduced body mass is evident as early as one month of age. The approach we have taken circumvents these kinds of problems, as normal constitutive expression levels are not disrupted. Nonetheless, this comparative approach is necessarily limited by inherent inaccuracies in the estimation of MLSP for individual species. These values are highly biased by the number of observations that can be made for each species: in most species MLSP estimates tend to be revised upward with increasing numbers of observations. Here we limited the negative effects of this issue by utilizing a relatively large collection of 13 species.

The positive correlation of Hsp levels with MLSP is interesting in light of our previous findings, using the same collection of vertebrate species, that other components of the protein homeostasis machinery show no correlation with lifespan (Salway et al., 2010). Levels of protein ubiquitylation do not show any relationship with species MLSP. Similarly, the activities of the 20S/26S proteasome and protein redoxins capable of reversing oxidative protein damage do not correlate with MLSP (Salway et al., 2010). Page et al (2010) found no evidence that antioxidant enzyme capacity is enhanced in long-lived species (Page et al., 2010). Thus, the positive correlation of protein folding and chaperone activities with MLSP appears to be somewhat unique.

Our results could shed light on those of Perez et al (2009) and Salmon et al (2009) who showed that proteins from longer-lived species are resistant to unfolding in an *in*

*vitro* assay. Although Perez et al indicated that Hsp70 and Hsc70 levels in liver were not different between short-lived mice and the long-lived naked mole rat, these data were not shown nor was information provided regarding the sequences of the naked mole rat proteins, which would be required to confidently conclude this. Therefore, it remains possible that higher levels of Hsp70, or of other Hsps not measured, might contribute to the resistance to protein unfolding observed in these studies. This seems intuitively more plausible than evolving enhanced structural stability via amino acid sequence changes in the entire protein complement. From an energetic standpoint, the evolution of greater Hsp levels to facilitate protein chaperoning, refolding or disaggregation might also be more favourable than enhancing the capacity to recycle damaged proteins via enhanced proteasomal activities, which would incur the costs of both degradation and re-synthesis. Both Perez et al (2009) and Salmon et al (2009) similarly demonstrated that 20S/26S proteasome activity in liver is not elevated in young adult naked mole rats or bats compared to mice. Taken together, the evidence suggests a broad evolutionary pattern of increased Hsp expression, rather than protein degradation, oxidative damage repair, or amino acid substitution (shown by Moosmann and Behl, 2008; Bender et al., 2008 for mtDNA encoded, but not nuclear encoded proteins) to improve protein homeostasis in longer-lived species. Of course, this does not preclude the evolution of novel strategies within particular species that may diverge from this general pattern.

All of the Hsps measured here are expressed both constitutively and inducibly. Therefore, it is possible that elevated levels of Hsp expression in longer-lived species is a result of higher cellular stress under basal conditions. Alternatively, it is possible that these individuals experienced more stress during the process of euthanization and tissue

excision. However, with the exception of deer which were taken by local hunters, conditions surrounding euthanization were strictly controlled for all animals used in this study. Perhaps most importantly, none of the animals were unduly stressed prior to euthanization and the time to tissue excision and freezing was similar in all circumstances. In any case, were such differences to be important they would most likely have increased variability of the data rather than introduced an artefactual trend, and correlation coefficients of the raw data were exceptionally high in most instances. Thus, the observed trends appear to reflect real differences in basal levels of Hsp expression associated with longevity that would facilitate improved protein homeostasis under steady state conditions. Although it is interesting to ask whether the longer-lived species from this study would also be better able to upregulate Hsp expression in response to an imposed stress, it was not practical to pursue this line of investigation in this study. This can, however, be investigated in cells that can be maintained and manipulated in culture, and we are currently studying skeletal myoblasts collected from the same species.

Although not all correlations of brain Hsp levels with MLSP were statistically significant, there was nonetheless a general pattern of higher Hsp levels in brain tissue from longer-lived species. This may provide an important insight into how longer-lived animals maintain normal brain function over their lifespans. Although we did not include human tissue in this study (because we could not adequately control time to tissue sampling), these results are likely to be relevant to humans. Many neurodegenerative diseases are classified as protein misfolding diseases, often characterized by accumulation of abnormal protein aggregates. Overexpression of Hsps has generally been associated with attenuation of disease severity, while reduced function increases

severity in a variety of models of mammalian neurodegeneration (McLean et al., 2002; Dou et al., 2003). While Hsp70 has been perhaps most studied in this context (Yoo et al., 1999; Dedmon et al., 2005), available evidence suggests similar roles for Hsp60 (e.g. Magen et al., 2008), Grp78 (Kudo et al., 2008), and Grp94 (Dukes et al., 2008). Thus, the co-evolution of increased basal Hsp expression with longevity suggests improved protein homeostasis in brain tissue and resistance to neurodegenerative disease in longer-lived species.

Although Hsp levels were generally correlated with species MLSP, there was no evidence that they were expressed at constitutively higher levels in long-lived Snell dwarf mice relative to normal littermates. This is consistent with the recent demonstration that Hsp mRNA levels in various tissues of Snell dwarf mice and growth hormone receptor-null mice are not broadly upregulated (Swindell et al., 2009). As mRNA levels often correlate poorly with protein levels (egs. Ideker et al., 2001; Le Naour et al., 2001), it was important to confirm the absence of a broad and systematic Hsp upregulation by direct quantification of protein levels. The lack of relationship is surprising. One characteristic of Snell dwarf mice is reduced IGF-1 signalling, which is relatively robustly associated with increased Hsp expression in invertebrates (Walker and Lithgow, 2003; Halaschek-Wiener et al., 2005). Similarly, a recent report (Stuart and Page, 2010) indicates that plasma IGF-1 levels are lower in larger mammalian species, which are typically longer-lived. This may contribute to the elevated expression of Hsps in longer-lived species. In any event, these divergent results suggest a different relationship between IGF-1 signalling status and longevity in mice than in invertebrates or an inter-species context. Further investigation will be required to understand why this is so.

In conclusion, our study shows strong correlations of Hsps with longevity in mammalian and avian species, suggesting that one mechanism underlying the evolution of longevity has been improved protein homeostasis via increased constitutive expression of Hsps. In contrast, we found no evidence for increased Hsp expression in tissues of long-lived Snell dwarf mice, suggesting no role for constitutive Hsp expression in this model of lifespan extension.

**Table 3.1.** Heat shock protein expression relative to rat for each species from three different tissues

| Species/Tissue      | HSP60       | HSP70       | GRP78       | GRP94       |
|---------------------|-------------|-------------|-------------|-------------|
| <b><u>Liver</u></b> |             |             |             |             |
| Mouse               | 0.905±0.133 | 0.869±0.214 | 0.914±0.045 | 1.205±0.070 |
| Rat                 | 1±0         | 1±0         | 1±0         | 1±0         |
| Cow                 | 1.598±0.251 | 3.846±0.571 | 1.490±0.109 | 2.80±0.249  |
| Pig                 | 1.389±0.096 | 2.709±0.423 | 1.303±0.098 | 2.867±0.54  |
| Rabbit              | 1.109±0.158 | 1.018±0.097 | 1.115±0.023 | 1.379±0.264 |
| Guinea Pig          | 1.308±0.157 | 2.084±0.338 | 1.244±0.071 | 1.724±0.085 |
| Sheep               | 1.290±0.430 | 2.444±0.357 | 1.274±0.074 | 2.209±0.413 |
| Squirrel            | 1.055±0.423 | 1.028±0.084 | 1.081±0.089 | 1.027±0.045 |
| Deer                | 1.293±0.264 | 2.455±0.502 | 1.174±0.049 | 1.519±0.020 |
| Gerbil              | 1.066±0.291 | 0.936±0.062 | 0.861±0.071 | 1.039±0.044 |
| Hamster             | 1.036±0.327 | 0.878±0.053 | 0.843±0.092 | 0.898±0.051 |
| Finch               | 1.144±0.129 | 1.080±0.193 | n.d.        | n.d.        |
| <b><u>Heart</u></b> |             |             |             |             |
| Mouse               | 0.768±0.252 | 0.364±0.030 | 0.986±0.169 | 0.969±0.083 |
| Rat                 | 1±0         | 1±0         | 1±0         | 1±0         |
| Cow                 | 1.349±0.183 | 5.345±0.696 | 1.654±0.076 | 2.06±0.076  |
| Pig                 | 1.156±0.201 | 2.059±0.474 | 1.336±0.045 | 2.68±0.436  |
| Rabbit              | 1.310±0.330 | 3.106±0.789 | 1.147±0.143 | 1.757±0.185 |
| Guinea Pig          | 0.939±0.121 | 1.840±0.369 | 1.211±0.112 | 1.967±0.26  |
| Sheep               | 1.657±0.351 | 3.116±0.610 | 1.434±0.244 | 2.005±0.116 |
| Squirrel            | 0.989±0.027 | 1.335±0.178 | 1.154±0.088 | 1.273±0.039 |
| Deer                | 1.517±0.439 | 3.332±0.093 | 1.289±0.115 | 1.682±0.13  |
| Gerbil              | 1.045±0.138 | 0.755±0.173 | 1.075±0.096 | 1.023±0.188 |
| Hamster             | 0.647±0.133 | 0.775±0.086 | 1.026±0.056 | 0.875±0.044 |
| Finch               | 1.269±0.401 | 1.166±0.137 | n.d.        | n.d.        |
| <b><u>Brain</u></b> |             |             |             |             |
| Mouse               | 0.875±0.244 | 0.874±0.106 | 1.144±0.462 | 1.058±0.114 |
| Rat                 | 1±0         | 1±0         | 1±0         | 1±0         |
| Cow                 | 1.789±0.201 | 1.372±0.108 | 1.405±0.269 | 1.692±0.216 |
| Pig                 | 1.997±0.088 | 1.906±0.294 | 1.460±0.255 | 2.145±0.342 |
| Rabbit              | 1.311±0.214 | 1.262±0.158 | 1.178±0.147 | 1.416±0.184 |
| Dog                 | 1.514±0.140 | 2.284±0.417 | 1.293±0.158 | 1.934±0.569 |
| Guinea Pig          | 1.433±0.146 | 1.861±0.274 | 1.409±0.219 | 1.401±0.11  |
| Sheep               | 1.30±0.056  | 1.338±0.398 | 1.228±0.080 | 2.053±0.202 |
| Squirrel            | 1.204±0.063 | 1.739±0.316 | 1.093±0.128 | 1.357±0.123 |
| Deer                | 1.389±0.035 | 1.307±0.064 | 1.164±0.069 | 2.375±0.370 |
| Gerbil              | 1.143±0.067 | 0.930±0.108 | 0.886±0.100 | 0.973±0.006 |
| Hamster             | 1.094±0.085 | 0.759±0.146 | 0.856±0.181 | 0.944±0.028 |
| Finch               | 1.149±0.136 | 1.231±0.165 | n.d.        | n.d.        |

n.d. = not determined

**Table 3.2.** Statistical analysis of linear regressions: Heat shock protein expression as a function of MLSP.

| Protein Level      | Correlation ( $R^2$ ) | Slope  | P-Value |
|--------------------|-----------------------|--------|---------|
| <b>Liver HSP60</b> |                       |        |         |
| Residual           | 0.5668                | 3.7098 | 0.002   |
| Residual + FIC     | 0.4644                | 2.6636 | 0.011   |
| <b>Heart HSP60</b> |                       |        |         |
| Residual           | 0.4583                | 1.3768 | 0.008   |
| Residual + FIC     | 0.3725                | 1.0472 | 0.023   |
| <b>Brain HSP60</b> |                       |        |         |
| Residual           | 0.3795                | 2.0464 | 0.013   |
| Residual + FIC     | 0.3598                | 1.496  | 0.022   |
| <b>Liver HSP70</b> |                       |        |         |
| Residual           | 0.3697                | 1.0826 | 0.020   |
| Residual + FIC     | 0.3516                | 0.995  | 0.027   |
| <b>Heart HSP70</b> |                       |        |         |
| Residual           | 0.3529                | 0.6493 | 0.021   |
| Residual + FIC     | 0.3174                | 0.8171 | 0.036   |
| <b>Brain HSP70</b> |                       |        |         |
| Residual           | 0.3772                | 0.6607 | 0.013   |
| Residual + FIC     | 0.3887                | 0.7246 | 0.015   |
| <b>Liver GRP78</b> |                       |        |         |
| Residual           | 0.3601                | 1.656  | 0.026   |
| Residual + FIC     | 0.3433                | 2.1436 | 0.038   |
| <b>Heart GRP78</b> |                       |        |         |
| Residual           | 0.4129                | 2.0904 | 0.017   |
| Residual + FIC     | 0.6394                | 3.149  | 0.003   |
| <b>Brain GRP78</b> |                       |        |         |
| Residual           | 0.3307                | 1.2457 | 0.042   |
| Residual + FIC     | 0.3300                | 0.9346 | 0.040   |
| <b>Liver GRP94</b> |                       |        |         |
| Residual           | 0.3315                | 0.6436 | 0.032   |
| Residual + FIC     | 0.4261                | 0.7512 | 0.020   |
| <b>Heart GRP94</b> |                       |        |         |
| Residual           | 0.6744                | 0.9672 | 0.001   |
| Residual + FIC     | 0.695                 | 1.3334 | 0.001   |
| <b>Brain GRP94</b> |                       |        |         |
| Residual           | 0.3001                | 0.9677 | 0.053   |
| Residual + FIC     | 0.3058                | 1.058  | 0.051   |



#### **Chapter 4: Increased basal Hsp levels and greater stress response in skeletal muscle myoblasts of longer-lived animals**

**Hypothesis:** Molecular chaperones play a role in conferring animal longevity in isolated myoblasts during unstressed and stressed conditions.

**Objective:** The primary objective of this project was to determine if protein degradation or refolding of proteins through molecular chaperones plays a role in animal longevity in unstressed conditions and to analyze the induction of these mechanisms during stressed conditions.

**Contributions:** I performed all experiments (except for cell culture), phylogenetic and statistical analysis and chapter write-up.

## 4.1 Introduction

Mammals are a very diverse class of animals with dramatically different physical characteristics, including a wide range of maximum lifespans (MLSP). This can range from three years to over a century in some cases. Interestingly, the aging process that is seen amongst mammals is remarkably similar, indicating common molecular and cellular mechanisms. However, there is limited understanding of these mechanisms and their determination remains a major goal. Furthermore, data from several studies has been published indicating that cellular stress resistance correlates positively with longevity (Kapahi et al., 1999; Kirkwood et al. 2000; Ogburn et al. 2001; Miller et al. 2010).

An important aspect to resistance to cellular stress is the maintenance of protein homeostasis. It has been shown that proteins are a vulnerable target of oxidative stress. Oxidative stress can lead to loss of a protein native structure which can reduce its activity. Furthermore, protein denaturation can result in protein aggregation, a feature of severe neurodegenerative diseases associated with aging (Reviewed in Ali et al., 2010). To maintain proper protein structure and stability, molecular chaperones or heat shock proteins (Hsps) are responsible for ensuring proper folding of newly synthesized or unfolded proteins and refolding of abnormal or damaged proteins (Reviewed in Bukau et al., 2006). Hsps have long been recognized as a possible player in organism aging and longevity, especially in *Caenorhabditis elegans* and *Drosophila* species. Overexpression of hsp-6 (Yokoyama et al., 2002) and hsp-16 (Walker and Lithgow, 2003) led to an extension of lifespan in *C. elegans*. A similar lifespan extension was seen in *Drosophila* that overexpressed Hsps (Morrow et al., 2010). However, overexpression of Hsp70 in healthy mice revealed no lifespan extension (Vanhooren et al., 2008). Furthermore, long-

lived dwarf or growth hormone/insulin-like growth factor-1 deficient mice revealed no global upregulation of Hsp mRNA levels (reviewed in Swindell 2009).

Hsps are a broad family of proteins, in which they can be constitutively expressed or inducible. For example, mitochondrial Hsp60 is present at basal levels and becomes upregulated in response to stress (Haynes et al., 2007). The heat shock response is an intracellular defence mechanism that has evolved to combat stressful conditions including heat shock, oxidative stress and UV radiation. Heat shock factor 1 (HSF-1) is a transcription factor that regulates the heat shock response through its actions on Hsp expression. It is ubiquitously expressed as a monomer and is maintained in an inactive state by association with Hsp70, Hsp90 and Hsp40. However, during stressful conditions, it separates from this complex, trimerizes and translocates to the nucleus to bind to heat shock response elements and facilitate transcription of Hsps (reviewed in Tower, 2008).

Although chaperones are capable of fully refolding proteins, many are irreversibly damaged and must be removed from the cell. Lack of removal can lead to their aggregation or interference in normal cellular functions (Grune et al., 2004). The 20S proteasome is a multi-subunit complex responsible for degradation of damaged proteins, especially those which have been affected by oxidative stress. Similarly to Hsps, results have demonstrated that the proteasome may play a role in animal longevity. For example, inhibition of proteasome activity results in an impairment of cell proliferation and shortening of lifespan in human fibroblasts (Torres et al., 2006). Previously, it was shown that 20S proteasome activity correlates negatively with body mass and metabolic rate most likely played a role in this result (Salway et al., 2010).

Satellite cells or muscle-specific stem cells are responsible for growth and repair of skeletal muscle. This is particularly important during aging due to the loss of regenerative capacity. Myoblasts are muscle precursor cells that have been committed to becoming skeletal muscle. In Chapter 3, it was found that Hsps were positively correlated with MLSP in animal tissues. However, a limitation of using tissue is that the animals may have been stressed prior to tissue harvest, creating biased results. The use of myoblasts allows for strict control of the environmental conditions, removing this potential bias. Another important advantage to the use of myoblasts is that one is able to subject the cells to stressors and analyze the inducibility of proteins.

In this study, we tested the hypothesis that longer-lived species have evolved stronger protein homeostasis defence mechanisms in the form of enhanced protein folding capacities and protein degradation capabilities. To address this, the activity of the 20S proteasome and protein levels of 4 different heat shock proteins were measured in isolated myoblasts of 8 mammalian species with MLSP ranging from 3 to 30 years. For the Hsps, we used antibodies raised to conserved epitopes to measure levels of mitochondrial Hsp60, ER GRP78 and GRP94, and cytosolic Hsp70. Through the use of this broad comparative approach, it is possible to avoid the assumption that any differences seen are attributable to MLSP and not body mass or another unknown trait. We used a residual analysis to remove any associations that body mass may have with our trait of interest. Furthermore, we also utilized Felsenstein's Independent Contrasts (FIC) to address any problems associated with the non-independence of data points that may arise due to shared phylogenetic ancestry. Finally, we also subjected the isolated

myoblasts to hydrogen peroxide to determine its effects on 20S proteasome activity and Hsp levels over a time course in 6 mammalian species.

## **4.2 Materials and Methods**

### **4.2.1 Materials**

Dulbecco's Modified Eagles' Medium (DMEM, high glucose), Ham's/F-10 Nutrient Mixture, Penicillin/streptomycin, ampicillin, gentamicin, nonessential amino acids, fetal bovine serum, and Trypsin were obtained from Hyclone (Logan, UT, USA). TrypLE<sup>TM</sup> Express cell dissociation reagent was obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were purchased from Bioshop (Burlington, ON, Canada), Fisher Scientific (Fair Lawn, NJ, USA) and Sigma Aldrich (Oakville, ON, Canada; including Fluka and Caledon). BioRad protein dye was purchased from BioRad Laboratories (Hercules, California, USA). Anti-Hsp60, anti-GRP94, anti-GRP78 and anti-Hsp70 primary antibodies were purchased from Abcam (Cambridge, Massachusetts, USA). Anti-rabbit and anti-goat secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, US). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA). Memcode reversible protein stain was purchased from Thermo Scientific (Rockford, Illinois, USA).

Young adult individuals of all species were used. Samples from livestock species (cow, pig, rabbit, goat and sheep) were collected either at the point of slaughter, or in unanesthetized individuals. Livestock species came from populations that were not treated with hormones. Mouse myoblasts were isolated from C57BL6 mice. Primary myoblasts from four of the rat samples were derived from Wistar rats. The remaining rat

myoblasts and canine myoblasts derived from limbic skeletal muscle were purchased as frozen cells at passage 2 from Cell Applications, INC (Table 1.1)(San Diego, CA, USA).

Table 4.1. Species from which myoblast cell lines were established.

| Common Name  | Species                      | Number of individuals (n) | Body Mass (g) | Muscle sampled | Source   |
|--------------|------------------------------|---------------------------|---------------|----------------|--|
| Mouse        | <i>Mus musculus</i>          | 2                         | 30            | Limbic         | One primary myoblast line from C57BL6 mice; One C2C12 cell line from ATCC                      |
| Rat          | <i>Rattus norvegicus</i>     | 5                         | 400           | Limbic         | Four primary myoblast lines from Wistar rats; One primary myoblast line from Cell Applications |
| Dog (Beagle) | <i>Canis familiaris</i>      | 1                         | 11000         | Limbic         | Cell Applications  |
| Goat         | <i>Capra hircus</i>          | 1                         | 61000         | Shoulder       | Local abattoir   |
| Sheep        | <i>Ovis aries</i>            | 1                         | 29500         | Shoulder       | Local abattoir   |
| Pig          | <i>Sus scrofa</i>            | 1                         | 100000        | Rib            | Local abattoir   |
| Rabbit       | <i>Oryctolagus cuniculus</i> | 1                         | 2600          | Thigh          | Local abattoir   |
| Cow          | <i>Bos Taurus</i>            | 2                         | 379500        | Shoulder       | Local abattoir   |

#### 4.2.2 Cell Culture: Animal Myoblasts

##### Non-Stressed Cells

Samples of muscle tissue from healthy young adult cow, mouse, sheep, goat, rabbit and pig were obtained from local abattoirs (see Table 1). All samples were collected from animals at the time of slaughter and were maintained on ice, in sterile PBS containing penicillin, streptomycin, ampicillin, and gentamicin for less than one hour. Connective tissue, blood vessels and fat were removed from the muscle, which was then rinsed in PBS containing penicillin, streptomycin, gentamicin, and ampicillin. Myoblasts were isolated from muscle tissue using a procedure modified from Barani et al. (2003). Briefly, the muscle tissue was minced and washed an additional 2 times in PBS containing antibiotics. Pronase (1mg/mL) was then added to the muscle samples at a ratio

of 2mL per 1 g tissue and the resulting suspension was incubated for 60 or 90 minutes at 37°C and was vigorously triturated at 10 minute intervals. Following incubation the solution was filtered through sterile cheesecloth and centrifuged at 60g for 3 minutes. The resulting supernatant was then centrifuged at 240g for 3 minutes. The supernatant was discarded and the pellet was resuspended in sterile PBS followed by centrifugation at 350g for 3 minutes. The pellet was then resuspended in growth media (Ham's/F-10 nutrient mixture containing FBS, non-essential amino acids, basic fibroblast growth factor, penicillin, ampicillin, streptomycin, gentamycin) and plated onto a collagen coated Petri dish. An additional volume of growth media was added two days after plating, and the media changed four days after plating. Myoblasts were evident on day four.

#### Stressed Cells

All myoblast cell lines were cultured as myoblasts for one to three passages at 3% O<sub>2</sub> and 5% CO<sub>2</sub> in a Thermo Forma series II water jacketed CO<sub>2</sub> incubator. Myoblasts were incubated in serum free media for 18 hours prior to exposure to hydrogen peroxide. The cells were then exposed to 25uM hydrogen peroxide for 6 hours, after which media was removed and refreshed. 3, 6 and 18h later cells were collected by scraping, and spun at 500g for 3 min, at 4°C. The cells were confirmed to be myoblasts based on their ability to differentiate into striated multinucleate myotubes that contracted spontaneously in culture.. Cell pellets were stored at -80°C.

#### ***4.2.3 Preparation of Whole Cell Lysates***

Lysates were prepared from cell pellets resuspended in ice-cold lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 40% glycerol, and 0.5% Nonidet P-40) and incubated for 1 h with periodic sonication (Ultrasonic Sonicator W-

375). Following incubation, cell lysates were centrifuged at 16,000 g, 4°C for 10 min (accuSpin Micro R; Fisher Scientific).

#### ***4.2.4 Quantification of Heat Shock Protein Levels***

Cell lysate proteins (10µg) were separated by SDS-PAGE and electrotransferred to a PDVF membrane. Even protein transfer was verified using Memcode reversible protein stain prior to immunoblotting. The membranes were then incubated in a blocking solution, consisting of 5% skim milk in 1x PBS, for 45 min at room temperature, and then probed with anti-Hsp60 (1:2500), anti-GRP78 (1:400), anti-Hsp70 (1:100) or anti-GRP94 (1:100) primary antibodies in 5% skim milk-1x PBS-T overnight at 4°C. After several washes, membranes were then probed with anti-rabbit or anti-goat secondary antibodies for 1 hour at room temperature, then washed and visualized using an Odyssey infrared visual system (LI-COR Biosciences). Protein level quantification was performed using Odyssey Imaging Software, Version 1.0. For each gel, band intensities were normalized to a sheep sample that served as an internal standard. To control for total protein transfer, Memcode stain was performed.

#### ***4.2.5 20S Proteasome Activity Assays***

Assays for 20S and 26S proteasome activities were performed as described by Rodgers and Dean (2003) at room temperature (23°C) using a Varian spectrofluorometer. Conditions for the 20S proteasome assay were 25mM HEPES pH 7.5, 0.5mM EDTA, 400µM peptide and 10µg sample. The peptide Suc-LEU-LEU-VAL-TYR was used to measure the chymotryptic activity of the proteasome. The assay was conducted with an excitation wavelength of 360nm and an emission detection wavelength of 460nm. Activities were determined by measuring the release of the AMC fluorophore from the



peptide-AMC complex for 15 min. Parallel assays were run with 0.2mM of the specific proteasome inhibitor MG-132 to determine non-proteasomal AMC release. This non-specific activity was minor, but was subtracted from the rate measured in the absence of inhibitor. The standard curve for proteasome calculations used a serial dilution of 8 $\mu$ M AMC calibration standard to obtain the relationship between arbitrary fluorescence units (AFU) and [AMC]. Specific proteasome activity was calculated by interpolation from the standard curve according to the equation:

Activity (pmol/hr/ $\mu$ g) = slope x (AFU/min) x conversion factor ( $\mu$ M/AFU) x vol x (60min/hr) per  $\mu$ g protein, where the conversion factor was 1/slope of the AMC standard curve.

#### ***4.2.6 Statistical Analyses***

Statistical analyses performed as in Chapters 2 and 3.

### **4.3 Results**

Hsp60 levels in the myoblasts of 8 species were determined through western blot. For each case, the data points were normalized to the sheep sample to account for variability between blots. A correlational analysis revealed a highly positive correlation ( $p < 0.05$ ) between MLSP and Hsp60 levels (Fig 4.1A). A residual analysis was performed to determine if this relationship was actually driven by body mass. This analysis yielded a weak positive correlation ( $p < 0.1$ ) between MLSP and Hsp60 levels. Another limitation to the comparative study is that all the data points are not independent due to their phylogenetic relatedness. To counter this limitation, a FIC analysis was

performed. The positive correlation ( $p < 0.05$ ) between MLSP and Hsp60 levels remained after this analysis (Table 4.3).

The same basic approach was used when measuring protein levels for the ER Hsps, GRP78 and GRP94. A strong positive correlation ( $p < 0.05$ ) was found between GRP78 levels and MLSP (Fig 4.1B). However, the correlation was weakened ( $p < 0.1$ ) after the residual and FIC analyses. Similarly, a positive correlation ( $p < 0.05$ ) was seen between GRP94 levels and MLSP (Fig 4.1C), which remained after residual and FIC analyses ( $p < 0.05$ ; Table 4.3).

For Hsp70, the same approach was taken to describe the relationship between protein level and MLSP. There was a strong positive correlation ( $p < 0.05$ ) between MLSP and Hsp70 levels (Fig 4.1D). Furthermore, this strong correlation ( $p < 0.05$ ) remained after residual and FIC analyses (Table 4.3).

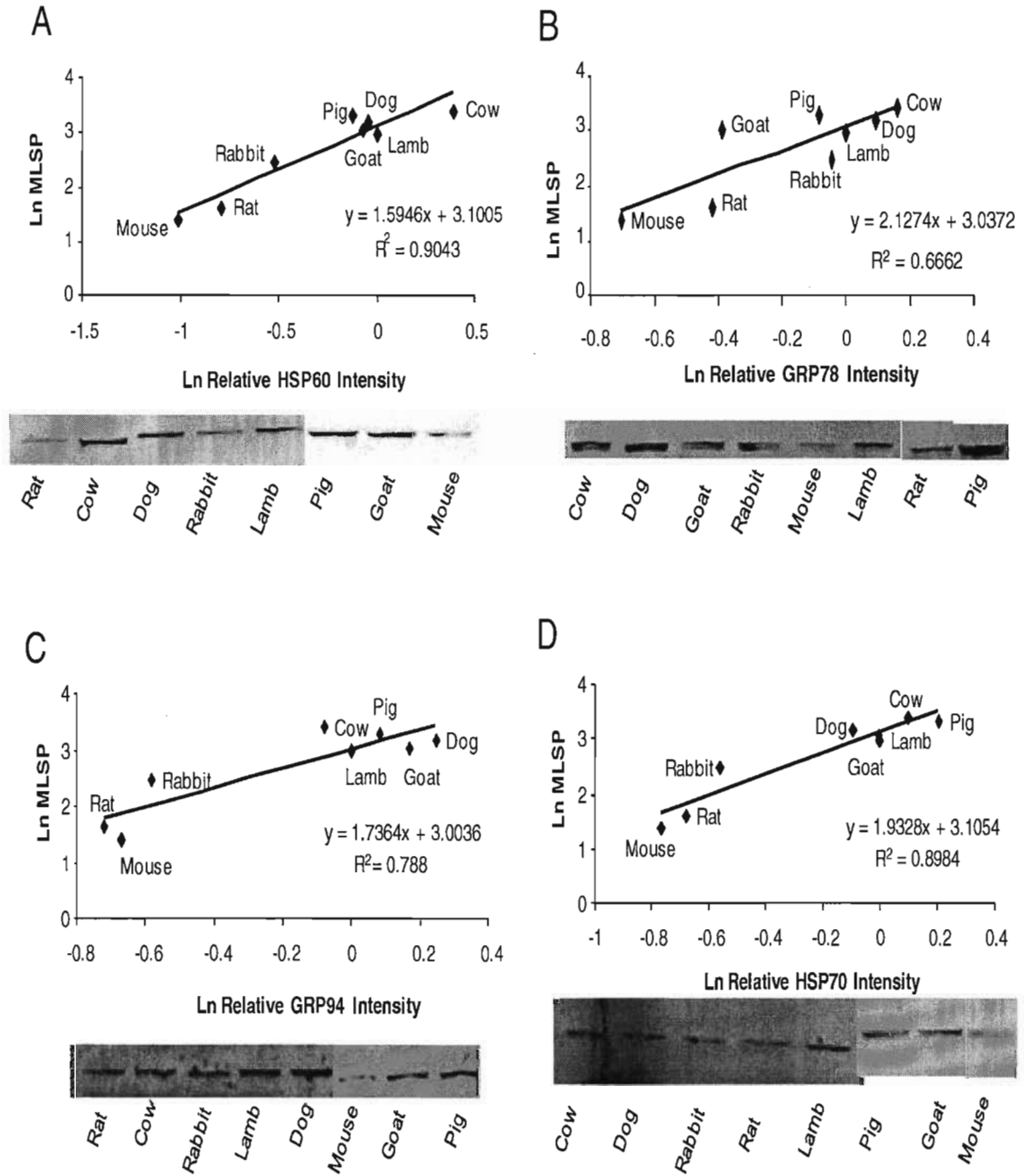


Figure 4.1. Correlations between Hsp protein levels and MLSP in isolated myoblasts. A)Hsp60, B)GRP78, C)GRP94 and D)Hsp70 protein levels were all positively correlated ( $p < 0.05$ ) with MLSP. All values are natural-log transformed.

To determine if protein degradative activities were correlated with animal longevity, we measured 20S proteasome activity in the myoblasts of all 8 mammalian species. However, there was no correlation between 20S proteasome activity and MLSP (Fig 4.2).

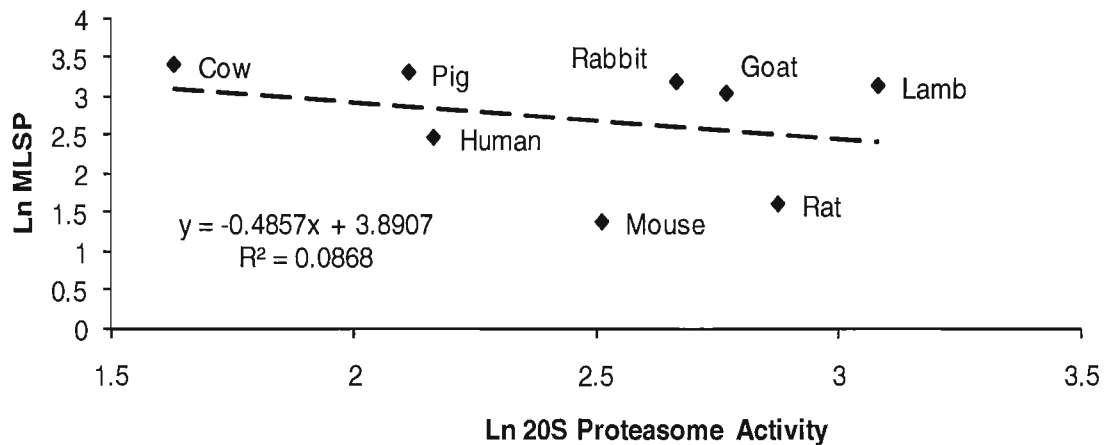


Figure 4.2. Correlation between 20S proteasome activity and MLSP in isolated myoblasts. No correlation was found between 20S proteasome activity and MLSP. All values are natural-log transformed and each data point represents the mean from duplicate measurements.

Hsp levels were measured in myoblasts of 6 mammalian species subjected to hydrogen peroxide to determine how a stressor effects the induction of each protein in each species. For each species, Hsp levels were determined at four different time points to determine peak induction. The peak induction of each protein and the time at which that peak induction occurred were each plotted with MLSP. A positive correlation ( $P < 0.05$ ) was found between MLSP and peak induction of Hsp60, GRP78 and GRP94 (Fig 4.3A-C). However, no correlation was found between Hsp70 peak induction and

MLSP (Fig 4.3D). For time of peak induction, a negative correlation was found between MLSP and Hsp60 ( $P<0.07$ ), GRP78 ( $P<0.1$ ) and Hsp70 ( $P<0.05$ ), suggesting that Hsps were induced more rapidly in longer-lived species. However, no correlation was found between MLSP and time to peak induction of GRP94 (Fig 4A-D).

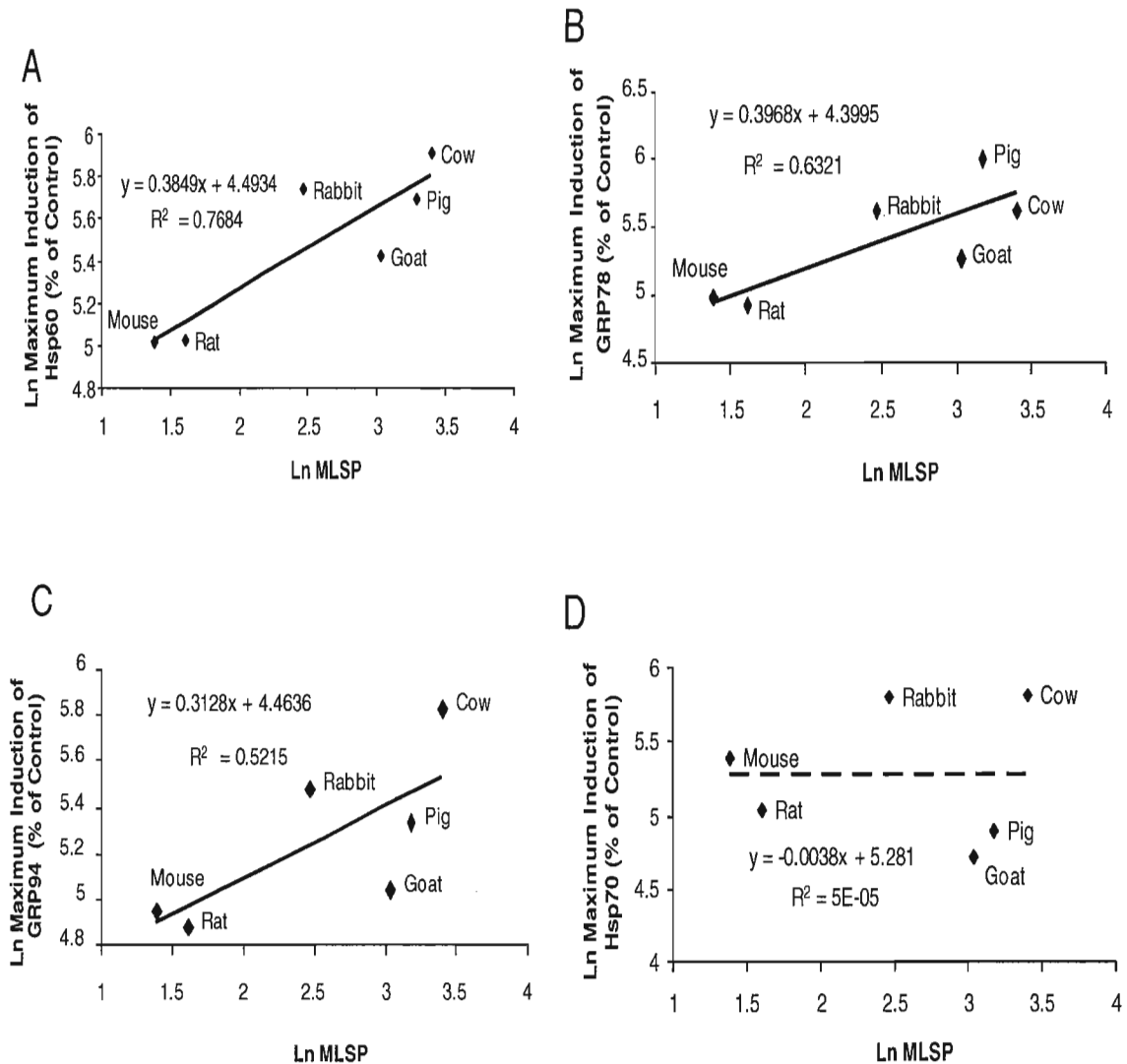


Figure 4.3. Correlations between maximum induction of Hsp protein levels and MLSP in isolated myoblasts. A)Hsp60, B)GRP78 and C)GRP94 protein levels were all positively correlated ( $p<0.05$ ) with MLSP. However, D)Hsp70 was not correlated with MLSP. All values are natural-log transformed.

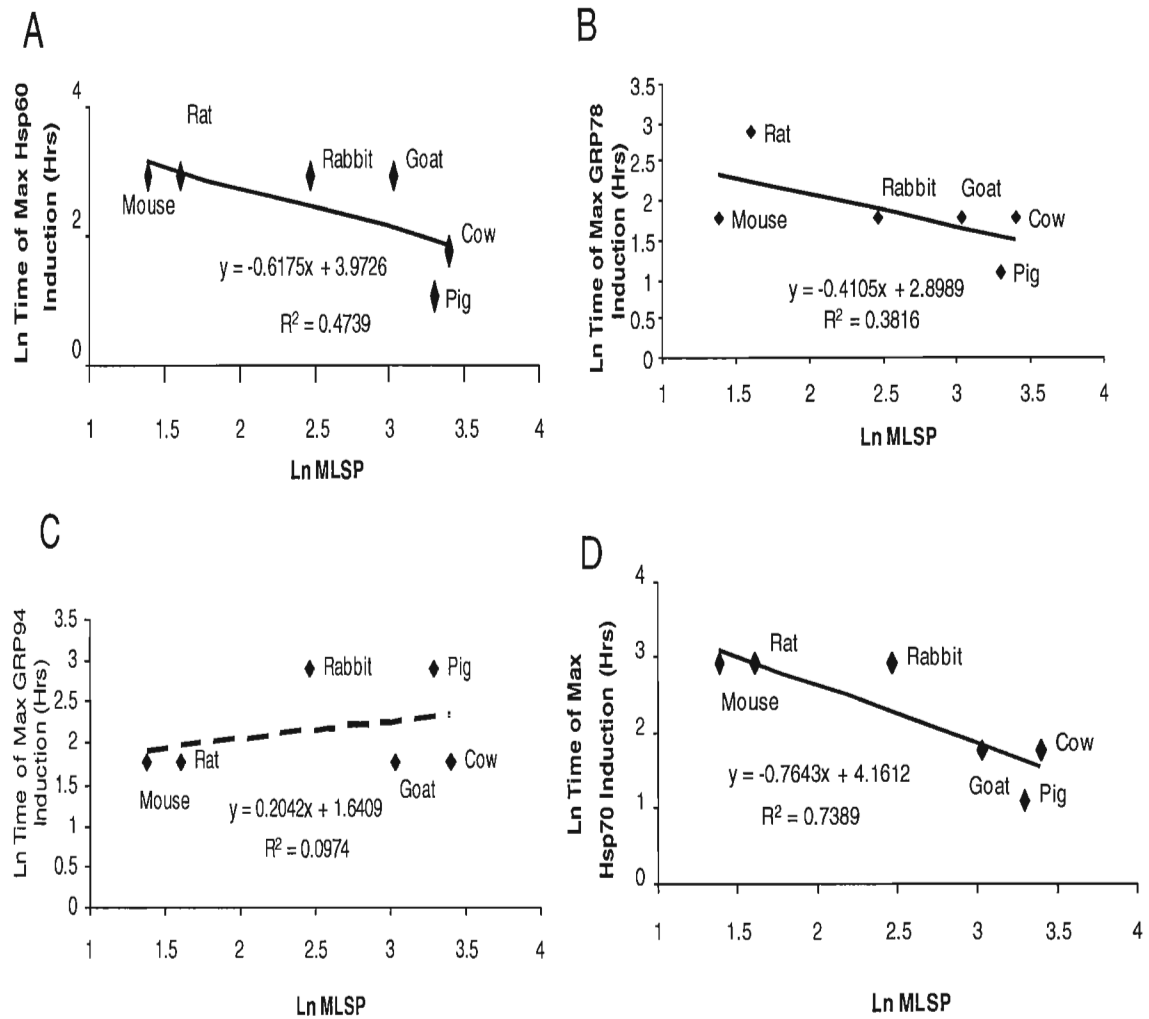


Figure 4.4. Correlations between time of maximum induction of Hsp protein levels and MLSP in isolated myoblasts. A)Hsp60 and B)GRP78 are positively correlated ( $p < 0.1$ ) with MLSP. C)GRP94 levels are not correlated with MLSP. D) Hsp70 protein levels are also positively correlated ( $p < 0.05$ ) with MLSP. All values are natural-log transformed.

20S proteasome activity was also measured in myoblasts under the same experimental conditions. However, no significant correlation was found between MLSP and peak

induction or time of peak induction for 20S proteasome activity (Fig 4.5A-B).

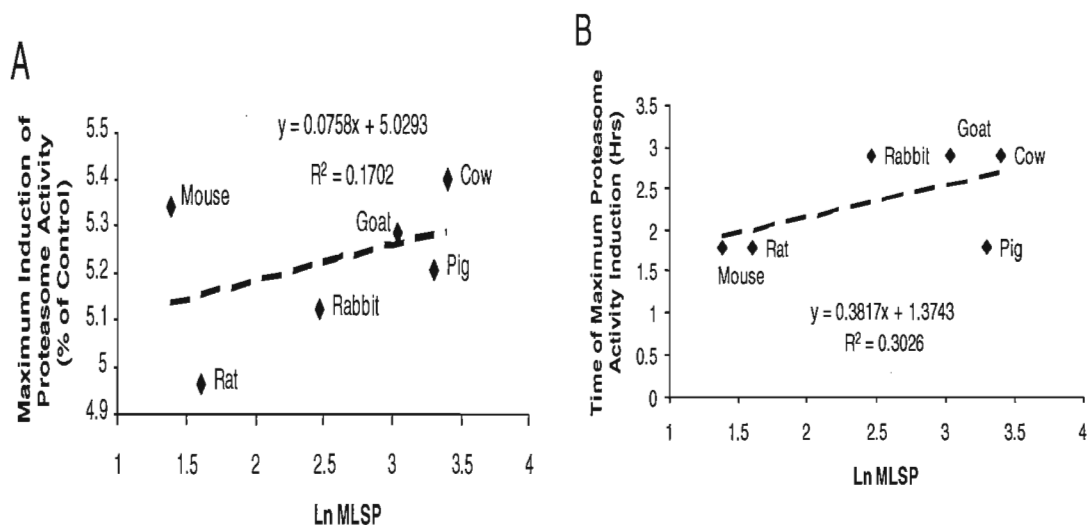


Figure 4.5. Correlation between maximum induction and time of maximum induction of 20S proteasome activity and MLSP in isolated myoblasts. There was no correlation between maximum proteasome activity or time to maximum induction and MLSP. All value are natural-log transformed.

#### 4.4 Discussion

Results from this study in isolated myoblasts indicate that basal Hsp expression levels are positively correlated with species' MLSP. This result is similar to those shown in Chapter 3, where Hsp expression levels were elevated in liver, heart and brain tissues of longer-lived species. Both results are consistent with published work from invertebrate models showing a strong relationship between enhanced Hsp levels and longevity (Yokoyama et al., 2002; Walker and Lithgow, 2003; Morrow et al., 2010). These inter- and intra-specific results suggest that Hsps play an important role in animal longevity.

Although a great deal of research has shown that overexpression of Hsps leads to lifespan extension in invertebrates, there is limited knowledge of this effect in mammals. Reported data includes the overexpression of Hsps in murine models of disease (e.g., amyotrophic lateral sclerosis), which increases in lifespan back to near-normal values (McArdle et al., 2004; McLean et al., 2002). However, overexpression of Hsp70 in healthy mice led to a shortening of lifespan, while increasing the incidence of tumour formation (Vanhooren et al., 2008). This suggests that global Hsp70 overexpression may negate the value of its protective function by promoting tumorigenesis through its anti-apoptotic capability (Vanhooren et al., 2008). This elevation of tumorigenesis is not observed in invertebrates because they are less prone to inflammation and cancer (Vanhooren et al., 2008). Thus, transgenic manipulations, particularly those that globally upregulate gene expression throughout development, can have unintended negative effects. The comparative approach used in this study can circumvent these problems. Nonetheless, there are limitations to this approach. These include inaccuracies in the



estimation of MLSP, and undue influence of individual species on correlations. Both problems can be overcome by including many species in the analysis. In the present study, both species number and the number of individuals sampled for each species was lower than is desirable, and further work should be done to verify the results from this smaller data set.

The Hsps measured in this study are expressed both constitutively and inducibly. The results in Chapter 3 were measured in animal tissues and that creates a limitation wherein the longer-lived species may have been more stressed under basal conditions or during euthanization. The use of the isolated myoblasts removes this limitation due to the strict control of their environmental conditions and energy levels. Furthermore, the use of myoblasts allowed us to understand if there were any differences in Hsp or proteasome activity upregulation in response to an imposed stress between the animals. This study found that Hsp expression in isolated myoblasts is correlated with species' MLSP, which suggests that stress, either inherent or during euthanization, may not have contributed to the observed trends in animal tissues (Chapter 3). Thus, the higher basal Hsp levels of longer-lived species appear to be based on genetic differences rather than resulting from stressful environmental conditions.

Heat shock transcription factors (HSFs) are a family of four transcription factors that regulate the expression of molecular chaperones. HSF-1 is the primary factor responsible for the cellular stress response. Most Hsp gene promoters (e.g. Hsp60 and Hsp70 promoters) contain a heat shock element (HSE) upstream of Hsp genes to which HSF-1 binds during stress and stimulates transcription of Hsps. However, its role in basal expression of Hsps has not been clearly identified. For example, HSF-1 expression

in yeast is essential for cell viability during unstressed conditions (Sorger, 1991). Contrastingly, HSF-1 is not required for cell viability in *Drosophila* (Jedlicka et al., 1997) or mammals (McMillan et al., 1998), and its deletion has no effect on the constitutive expression of Hsps in mammals. Therefore, HSF-1 activity appears to play no role in constitutive expression of Hsps.

However, another member of the HSF family, HSF-4, has been observed to regulate the basal expression of Hsps. It exists as two different isoforms, where HSF-4a is a repressor of basal expression of Hsps through its interaction with the basal transcriptional machinery (Frejtag et al., 2001). Overexpression of HSF-4 leads to a repression of target genes that are regulated through HSE promoter sequences (Nakai et al., 1997). Thus, HSF-4 binds the HSE sequence and interacts with TFIIF, a transcription factor required for basal transcription, leading to repression of Hsp expression (Frejtag et al., 2001). Therefore, the differential regulation of basal Hsp expression in mammals may be due to HSF-4 and it is possible that longer-lived species have evolved to produce less of this protein during unstressed conditions, leading to increased levels of Hsps.

Since HSF-1 is the primary transcription factor responsible for stimulating Hsp expression during stressful conditions, it is possible that there are elevated levels of HSF-1 or post-translational modifications to it have evolved in longer-lived animals to create a superior stress response. However, these possible mechanisms have not been shown in longer-lived species. Our study found that for the majority of the Hsps, longer-lived animals had a greater and more rapid induction of Hsp expression levels. Therefore, this may indicate some evolutionary change that facilitates faster and greater stress response in longer-lived animals. HSF-1 also undergoes numerous post-translational

modifications that regulate its DNA-binding activity and transcriptional activity. These modifications could lead to a greater and more rapid stress response in longer-lived animals. HSF-1 contains several serine amino acids that can be phosphorylated. For example, Ser230 phosphorylation is elevated during stressful conditions, leading to greater activation of HSF-1 (Holmberg et al., 2001). Furthermore, stress-induced SUMO modification of HSF-1 increases the transcriptional activity of HSF-1 (Hong et al., 2001). Acetylation also plays a large role in activation of HSF-1 during stress. Acetylation of HSF-1 at lysine residues negatively regulates its DNA binding activity. However, the deacetylase SIRT1 will deacetylate HSF-1 and promote its binding to DNA and increase transcriptional activity (Westerheide et al., 2009). Therefore, post-translational modifications of HSF-1 leading to increased transcriptional activity may explain the greater and more rapid induction of the heat shock response in the longer-lived animals.

The genes encoding endoplasmic reticulum specific chaperones, GRP78 and GRP94, do not contain heat shock elements and hence, are regulated through a HSF-independent pathway. However, these proteins do contain an ER stress response element (ERSE) which appears to act similarly to the HSE (Yoshida et al., 1998). Furthermore, the transcription factor ATF6 is able to activate the transcription of GRP genes constitutively and inducibly, similarly to the HSFs. It is also been suggested that ATF6 is regulated by post-translational modifications (Zhu et al., 1997). Therefore, this pathway may have evolved to increase its activities at basal and stress conditions in longer-lived species.

The 20S proteasome is responsible for the degradation of damaged proteins. Previously, it was shown that in animal tissues, 20S proteasome activity correlates

negatively with MLSP but that correlation was driven by body mass (Salway et al., 2011). However, our results in myoblasts suggest that there is no correlation between proteasome activity and MLSP or body mass. One possible explanation for these differences involves mass-specific metabolic rate. It has been shown that there is a negative correlation between mass-specific metabolic rate and MLSP (Hulbert et al., 2007). Proteasome activity may be regulated by metabolic rate, and when mass-specific metabolic rate decreases so does its activity. However, the same correlation between mass-specific metabolic rate and MLSP was not seen in isolated myoblasts (unpublished data, Robb and Stuart, 2011). Therefore, one would not expect a correlation of proteasome activity and MLSP or body mass under culture conditions

In conclusion, this study shows strong correlations between Hsps and mammalian lifespan, which suggests that enhanced protein homeostasis due to an elevation in basal Hsp expression levels has co-evolved with longevity in this clade. However, there was no enhancement of 20S proteasome activity in longer-lived animals. Furthermore, myoblasts of longer-lived animals subjected to oxidative stress showed a greater and more rapid induction of Hsps, indicating a more superior stress response.

Table 4.2. Basal Heat shock protein expression relative to lamb for each species from isolated myoblasts

| <b>Species</b> | <b>Hsp60</b> | <b>GRP78</b> | <b>GRP94</b> | <b>Hsp70</b> |
|----------------|--------------|--------------|--------------|--------------|
| Mouse          | 0.3648       | 0.4958       | 0.5126       | 0.4651       |
| Rat            | 0.5545       | 0.6572       | 0.4880       | 0.5061       |
| Cow            | 1.2894       | 1.1730       | 0.9249       | 1.1014       |
| Pig            | 0.9443       | 0.9194       | 1.3296       | 1.0877       |
| Lamb           | 1            | 1            | 1            | 1            |
| Rabbit         | 0.5929       | 0.9560       | 0.5595       | 0.5709       |
| Goat           | 0.9349       | 0.6787       | 1.1841       | 0.9942       |
| Dog            | 0.9552       | 1.0972       | 1.2817       | 0.9087       |

Table 4.3. Statistical analysis of linear regressions: Heat shock protein expression as a function of MLSP.

| <b>Protein Level</b> | <b>Correlation (<math>R^2</math>)</b> | <b>Slope</b> | <b>P-value</b> |
|----------------------|---------------------------------------|--------------|----------------|
| <b>Hsp60</b>         |                                       |              |                |
| Residual             | 0.2619                                | 0.8504       | 0.097          |
| Residual + FIC       | 0.4688                                | 1.3646       | 0.045          |
| <b>GRP78</b>         |                                       |              |                |
| Residual             | 0.2343                                | 0.6074       | 0.112          |
| Residual + FIC       | 0.3074                                | 0.8269       | 0.098          |
| <b>GRP94</b>         |                                       |              |                |
| Residual             | 0.5241                                | 0.725        | 0.021          |
| Residual + FIC       | 0.7204                                | 0.9087       | 0.008          |
| <b>Hsp70</b>         |                                       |              |                |
| Residual             | 0.3903                                | 1.0207       | 0.049          |
| Residual + FIC       | 0.4756                                | 1.3873       | 0.043          |

## **Chapter 5**

### **5.1 General Discussion**

Cellular stress resistance seems to play a key role in animal lifespan. Several approaches have been taken to examine stress resistance in different animals. Results from inter-specific (Kapahi et al., 1999) and intra-specific (Salmon et al., 2005; Murakami et al., 2003; Harper et al., 2007) studies have shown that an increase in stress resistance is associated with increased longevity. This suggests that longer-lived species or strains have enhanced mechanisms for maintaining cellular homeostasis. While these mechanisms have not been elucidated, it has been suggested that longer-lived species have enhanced protection from stressors due to superior maintenance of cellular homeostasis. Proteins are susceptible to numerous stressors including oxidative and thermal stress. Exposure to these stressors can directly alter protein structure, leading to decreases in activity and aggregation of denatured proteins. Therefore, possible mechanisms responsible for maintaining protein structure and function could include enhanced protein degradation, protein repair and/or protein chaperoning.

### **5.2 Protein turnover and body mass**

Two potential mechanisms for maintaining protein homeostasis and conferring animal longevity are protein degradation and protein repair. In this thesis, I hypothesized that enhanced activities of protein degradation via the proteasome and protein repair through thioredoxin reductase and glutaredoxin would be associated with increased stress resistance and lifespan. However, I found no correlations between either protein degradation (proteasome) or protein repair (redoxins) activities and MLSP. Rather, there

were actually significant negative correlations between body mass and protein degradation or repair activities in some tissues. Previous studies have shown that basal metabolic rate decreases with increasing body mass, which can contribute to similar decreases in protein turnover with increases in species size (Regoeczi and Hatton, 1980). Therefore, the negative correlations observed between protein degradation and protein repair activities and body mass may be linked to decreases in basal metabolic rates and protein turnover rates in larger animals.

To determine if a similar trend exists in cell culture, protein degradation activity was measured in isolated myoblasts, where it was found that there was no correlation between proteasome activity and either MLSP or body mass. Previously, it was found that myoblasts from different species all adopt the same basal metabolic rate in culture (Robb and Stuart, unpublished data). Therefore, if protein turnover is linked to basal metabolic rate and this normalizes in culture, it is not surprising that no correlation between proteasome activity and species body mass exists in this context.

### **5.3 Evidence for a role of molecular chaperones in vertebrate longevity**

Enzymes involved in folding proteins into their native structure play an integral role during normal cellular function and in response to stressful environmental conditions. Overexpression of heat shock proteins (Hsps) have been shown to extend lifespan in the invertebrate animal models *C. elegans* and *D. melanogaster*. In this thesis, I hypothesized that the expression of molecular chaperones would be enhanced in longer-lived species. To evaluate the effects that molecular chaperones had on MLSP in vertebrates, basal protein levels of 4 different heat shock proteins were measured in 15

different mammals and birds in liver, heart and brain tissues. Positive correlations between MLSP and Hsp levels were identified in all three tissues, suggesting that Hsps may indeed contribute to the increased stress resistance that appears to have co-evolved with longevity.

In animal tissues, the environmental conditions to which individual cells are exposed cannot be fully controlled, so it is possible that higher Hsp levels are caused by greater stress either chronically or during tissue harvest. By repeating these measurements in cultured myoblasts, I was able to address this issue, as conditions in culture are strictly controlled and identical for cells of all species. Basal protein levels of the same four Hsps were measured in isolated myoblasts from 8 mammals, and showed similar positive correlations between MLSP and Hsp levels. This suggests that there is a genetic component driving the Hsp correlations seen between species. It also further suggests a role for molecular chaperones in cellular stress resistance and animal lifespan.

Another advantage of experimentation in a cell culture environment is the ability to expose cells to stress and examine the induction of stress-response proteins such as the Hsps. To identify the magnitude of the stress response between species, the maximal induction and time to maximum induction of the Hsps was measured in 6 different species. Interestingly, positive correlations were observed between maximal Hsp induction and MLSP, and negative correlations between time to maximal Hsp induction and MLSP for most of the individual Hsps. These results further suggest that more robust Hsp expression associated with increased species longevity contributes to the observed differences in cellular stress resistance.



#### **5.4 Molecular mechanisms of stress resistance in the Snell dwarf mouse**

Long-lived mutant mice, including the Snell dwarf, are well known to be more stress resistant than their normal littermates (Murakami et al., 2003; Salmon et al, 2005; Maynard and Miller, 2006). Therefore, a major goal of this thesis was to identify cellular mechanisms for increased stress resistance in these long-lived mice. Surprisingly, my results provide no evidence that maintenance of enhanced protein degradation or protein repair activities in heart or brain tissues over the adult lifespan contributes to Snell dwarf mouse longevity. Rather, many of these activities including the 20S proteasome, TrxR and Grx were actually lower in Snell dwarf than normal mice (Chapter 2). Similarly, there was no evidence for a role of Hsps in Snell dwarf longevity (Chapter 3), a result that is supported by Hsp mRNA measurements of Swindell et al. (2009) made in the Snell dwarf mouse under similar conditions. These results therefore do not support a role for enhanced protein homeostatic mechanisms in the longevity of Snell dwarf mice.

#### **5.5 Protein homeostasis in mitochondria**

Mitochondria are the main source of ROS, the primary endogenous agent causing oxidative stress. Their proximity to ROS requires that they have their own mechanisms for removing damaging molecules. Mitochondria also possess their own protein homeostasis mechanisms responsible for maintaining protein structure and function within the mitochondria. Therefore, it is possible that cellular stress resistance can be achieved through superior protein homeostasis mechanisms within the mitochondria. Protein degradation, repair and chaperoning are accomplished through proteins that are found throughout the cell in different subcellular compartments. Thus, isoforms of

proteins responsible for protein homeostasis are found in the cytosol and the mitochondria. In this thesis, many of the measurements were done on whole cell homogenates from animal tissues, so it cannot be determined in all cases which subcellular compartments contribute to the trends observed.

Similarly to the cytosol, the mitochondria contain their own protein degradation mechanism, which is important for degrading oxidatively damaged proteins within the mitochondria. The Lon protease is a mitochondria-specific protease that is responsible for the majority of protein degradation in the mitochondria. Downregulation of Lon protease leads to impaired mitochondrial function and apoptosis (Bota et al., 2005). Furthermore, when faced with stress, Lon protease levels are elevated and its downregulation can lead to an increase in protein carbonyl damage (Ngo and Davies, 2009). Therefore, the activity of these mitochondria-specific proteases, especially the Lon protease, also may play an important role in conferring stress resistance and longevity.

The mitochondria also contain isoforms of some of the protein repair enzymes measured here, including TrxR and Grx. Mice with a partial deficiency of TrxR-2 (mitochondrial) have been shown to have a decrease in mitochondrial function and have proteins more susceptible to oxidative damage (Perez et al., 2008). Furthermore, there are other protein repair enzymes found throughout the cell including thioredoxin (Trx) which contains cytosolic and mitochondrial isoforms. Overexpression of Trx-1 has been shown to increase lifespan in mice (Mitsui et al., 2002). Therefore, it appears that isoforms of protein repair enzymes responsible for protein redox homeostasis found in the mitochondria may be key players in animal longevity. The extent to which

mitochondrion-specific redoxin activities might have contributed to my results is not known, and addressing this question would require first isolating mitochondria from all tissues.

Mitochondria must also contain their own machinery for properly folding newly transported proteins or refolding damaged proteins. Therefore, they possess their own molecular chaperones. There are numerous heat shock proteins including Hsp60, mt-Hsp70 and other small Hsps (eg. Hsp10). In Chapter 3 and 4, it was found that Hsp60 levels were higher in longer-lived animals, and it exhibited a greater stress response in longer-lived animals. Overexpression of Hsp60/Hsp10 leads to protection against ischemic injury in rat cardiac myocytes. This evidence suggests that Hsp60 is important in stress resistance and animal longevity. Therefore, other mitochondrion-specific chaperones may be similarly valuable in maintaining mitochondrial protein structure and function, and reducing the effects of oxidative stress.

## **5.6 Regenerative stem/progenitor cells**

Tissue resident stem cells are pluripotent cells that are able to differentiate into different cell types within an organ. These cells constitute a small percentage of the overall population of cells within different tissues. Recently, the maintenance of adult stem cells has been shown to be important in cellular maintenance and animal aging. Furthermore, stem cells do age and can stop functioning as regulators of cellular maintenance (reviewed by Mantel and Broxmeyer, 2008). Therefore, longevity may be conferred through mechanisms allowing the maintenance of robust stem cells for a longer period of time.

In this thesis, myoblasts, the skeletal muscle precursor cells, were used as representative stem/progenitor cells to test if the cells of longer-lived animals were more stress resistant and robust. In Chapter 4, I found positive correlations between Hsp levels and MLSP in isolated myoblasts of longer-lived species. This suggests that progenitor cells of longer-lived species may have become more stress resistant through an increase in molecular chaperone protein levels. Previous studies have shown that proliferative embryonic stem cells do have higher levels of molecular chaperones (Prinsloo et al., 2009). However, there did not appear to be correlations between proliferation rate and species' MLSP in the myoblasts (Robb and Stuart, unpublished data), suggesting the trend was not driven by differences in proliferation rate. It would be interesting to determine if similar trends existed in progenitor cells from the highly oxidative tissues like heart and brain, especially since they are particularly prone to age-related diseases.

## **5.7 Conclusions**

Based on the results of this thesis, I make three conclusions regarding the roles of protein repair, protein degradation and Hsp-mediated chaperoning in longevity. Firstly, it appears that there is no evidence that protein degradation, repair or molecular chaperoning contributes to longevity in Snell dwarf mice. Secondly, there is no evidence that either protein degradation or protein repair correlate with longevity of vertebrate species. Thirdly, strong positive correlations between Hsp protein levels and MLSP were identified, and these manifested in the major oxidative tissues and also in isolated myoblasts. In addition, it was also found that Hsps are generally more rapidly and strongly induced in response to hydrogen peroxide stress in myoblasts from longer-lived

species. These results strongly suggest that longer-lived species have a more robust protein chaperoning capacity that may contribute to their longevity.

Future research may be directed towards understanding how mechanisms of protein homeostasis could contribute to prevention of age-related diseases, in particular neurodegenerative diseases. Understanding the mechanisms that underlie animal longevity could lead to more comprehensive knowledge of the aging process. Potentially, this could lead to slower aging and longer lifespans.

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